Histidine - P. 25

JANUARY 10, 1941

THE JOURNAL OF NUTRITION

VOLUME 21 NUMBER 1

GEORGE R. COWGILL, *Editor*
Yale University School of Medicine, New Haven, Conn.

WILLIAM H. CHAMBERS, *Assistant to the Editor*
Cornell University Medical College, New York City

ASSOCIATE EDITORS

JOHN B. BROWN — ERNEST B. FORBES — HENRY A. MATTILL
L. H. NEWBURGH — PHILIP A. SHAFFER — HAROLD H. MITCHELL
ALBERT G. HOGAN — ICIE MACY-HOOBLER — LELA E. BOOHER
HOWARD B. LEWIS — ALFRED T. SHOHL

Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.

Price, $5.00 per volume; Domestic; $5.50 per volume, Foreign; $6.25 per volume in Canada

Entered as second-class matter January 20, 1934, at the post office at Philadelphia, Pa., under Act of March 3, 1879. Copyright 1940, The Wistar Institute of Anatomy and Biology. All rights reserved.

Publications of The Wistar Institute

JOURNAL OF MORPHOLOGY

Devoted to the publication of original research on animal morphology and physiology, including cytology, protozoology, and the embryology of vertebrates and invertebrates. Articles do not usually exceed 50 pages in length.

Issued bimonthly; 2 vols. annually; $20.00 Domestic, $21.00 Foreign, $23.50 Canadian per year.

THE JOURNAL OF COMPARATIVE NEUROLOGY

Publishes the result of original investigations on the comparative anatomy and physiology of the nervous system.

Issued bimonthly; 2 vols. annually; $15.00 Domestic, $16.00 Foreign, $18.00 Canadian per year.

THE AMERICAN JOURNAL OF ANATOMY

Devoted primarily to the interests of anatomy as taught in the medical schools of the United States and to the development of scientific anatomy in these schools. Publishes researches only.

Issued bimonthly; 2 vols. annually; $15.00 Domestic, $16.00 Foreign, $18.00 Canadian per year.

THE ANATOMICAL RECORD

Organ of the American Association of Anatomists and the American Society of Zoologists

For the prompt publication of concise original articles on vertebrate anatomy, preliminary reports; technical notes; critical notes of interest to anatomists and short reviews of noteworthy publications.

Issued monthly; 3 vols. annually; $22.50 Domestic, $24.00 Foreign, $27.00 Canadian per year.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

Publishes papers embodying the results of original researches of an experimental or analytical nature in the field of zoology.

Issued 9 times a year; 3 vols. annually; $22.50 Domestic, $24.00 Foreign, $27.00 Canadian per year.

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

In this journal appear original papers on anthropology in general; researches in evolution, man's origin; human ontogeny; variations in man; heredity; eugenics; demography; the American Indian and abnormal classes. Papers on anthropometry and the methods of anthropology also appear.

Issued quarterly; 1 vol. annually; $6.00 Domestic, $6.50 Foreign, $7.25 Canadian per year.

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

Publishes papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects.

Issued bimonthly; 2 vols. annually; $10.00 Domestic, $11.00 Foreign, $12.50 Canadian per year.

THE JOURNAL OF NUTRITION

Organ of the American Institute of Nutrition

Publishes original researches in the field of nutrition and occasional reviews of literature on topics with which the journal is concerned.

Issued monthly; 2 vols. annually; $10.00 Domestic, $11.00 Foreign, $12.50 Canadian per year.

THE AMERICAN ANATOMICAL MEMOIRS

Publishes original monographs based on experimental or descriptive investigations in the field of anatomy which are too extensive to appear in the current periodicals. Each number contains only one monograph. List of monographs already published, with prices, sent on application.

ADVANCE ABSTRACT CARD SERVICE

Every paper accepted for publication in one of the above periodicals is accompanied by the author's abstract. The abstract and the complete bibliographic reference to the paper as it will eventually appear is printed on the face of a standard library catalogue card. This Advance Abstract Card Service is issued promptly, in advance of the journal containing the paper, and is offered in three styles.

Prices per year $2.00, $2.50 and $3.00, postpaid.

These publications enjoy the largest circulation of any similar journals published.

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

Woodland Avenue and Thirty-sixth Street, Philadelphia, Pa.

EDITORIAL REVIEW

THE BIOASSAY OF VITAMIN K*

S. ANSBACHER

Division of Experimental Medicine, The Squibb Institute for Medical Research, New Brunswick, New Jersey

(Received for publication August 1, 1940)

In vitamin research, rapid progress is made possible by accurate methods of detecting and measuring the active principle. In investigations on vitamin K, biological procedures have been relied upon almost exclusively and, as with other vitamins, they were of the preventive or curative categories.

PREVENTIVE BIOASSAYS

In the early studies from 1929 to 1935, the preventive type of bioassay was solely employed. Dam ('35) assayed foodstuffs by rearing chicks for 1 month on a vitamin K-deficient ration in which the test material had been incorporated. If the blood clotting times of the animals fed this diet were considerably shorter than those of the controls receiving the deficient ration, then the material was considered to contain vitamin K.

Almquist and Stokstad ('37 b) adopted Dam's method, shortened the test period to 2 weeks, and used clotting times of more than 30 minutes as criterion for a negative result. This technic was modified by Almquist et al. ('38) in that chicks were first reared on a deficient diet (Almquist and Stokstad, '37 a) for 1 week, then from 7 to 14 or 21 days on the supplemented ration. The assay was terminated after

* A part of the material in this paper was presented before the Chemistry Section of the American Association for the Advancement of Science, Gibson Island, Maryland, July 16, 1940.

THE JOURNAL OF NUTRITION, VOL. 21, NO. 1
JANUARY, 1941
Copyright 1940.
The Wistar Institute of Anatomy and Biology.
All rights reserved.

7 days of supplementary feeding, when a series of suitable groups was available receiving the "Almquist reference standard," a hexane extract of alfalfa to which no "unit" value was assigned.

After attempts to shorten the assay time had failed, Almquist and Klose ('39 a) described a third method, in which chicks were kept for 2 weeks on the supplemented diet following the initial week on the deficient ration, although spontaneous cures had been observed to occur frequently during the third week. Moreover, the simple clotting test was replaced by Quick's ('38) prothrombin determination showing less variability when carried out with whole blood than with plasma. However, according to Macfie et al. ('39), "determination of crude blood-clotting times appears to offer a better means of biological assay than determination of prothrombin times, contrary to the recent findings of Almquist and Klose."

The preventive method of Almquist and Stokstad ('37 b) was employed by Dann ('38) whose unit was described by Snell ('39): The Dann unit is the least amount per gram of a chick's body weight daily which is necessary to prevent prolongation of clotting time beyond normal limits during a 2-week assay period. Dann noted that less than 50% of her negative controls had markedly prolonged clotting times and that the incidence of hemorrhage in these groups was less than 20%. In order to minimize the influence of these adverse conditions, a clotting time of less than 1 minute was considered normal, although it may be even longer than 6 minutes (Schønheyder, '36 a; Cheney, '39). Later, Dann ('39) followed the example of Almquist and Klose ('39 d) by adopting an assay based upon a curative technic; thus, all investigators confirmed Schønheyder's ('36 b) result: "Vitamin K is best estimated by the curative method."

CURATIVE BIOASSAYS

The first curative bioassays were made by Schønheyder ('36 b) with a technic based upon the observation that "animals suffering from vitamin K deficiency became normal with

reference to clotting time in 3 days when given sufficient food containing vitamin K." The coagulation process was determined by a method similar to that of Quick ('38), but instead of measuring it as prothrombin time, the relation between the concentrations of the added clotting agent, which coagulate a plasma from a sick animal and normal plasma in 3 minutes at 40°C., was considered as index of the degree of deficiency. The Schønheyder unit of vitamin K is defined as the smallest daily dose of a test substance per gram of chicken for 3 days which is able to reduce the animal's S value, i.e., the degree of sickness, from over 1500 to 10. This unit is small, but when it is multiplied by 1000 it equals the amount of substance which is necessary to cure an animal with an S value of 1500 to 2000 and a weight of 333 gm. in the course of 3 days, the animal receiving one-third every day.

Schønheyder's method was revised by Dam and Glavind ('38) who determined the degree of sickness of the individual animals prior to the assay. The Dam unit of vitamin K is defined as that amount which is to be administered to the test animal per gram of body weight in order to reduce its value $R \geqq 200$ to 1, $R = \frac{K}{K_n}$; K equals concentration of the tissue extract necessary to cause clotting of the blood plasma in 3 minutes; K_n equals the corresponding concentration for normal plasma. Apparently Dam's "R" value (Dam and Glavind, '40) is the same as Schønheyder's "S" value divided by 10; it characterizes the degree of reduction of clotting power and is roughly 1 for normal chicks. Furthermore, a selected dried spinach powder, formed into tablets and stored under precautions securing stability, was chosen as a standard and found to contain 500 Dam units per gram.

Thayer et al. ('38) modified Schønheyder's assay by replacing the complicated coagulation technic with the simple clotting test. Arbitrarily, 10 minutes or less was considered normal, although earlier data (Thayer et al., '37) had indicated a range of 1 to 6 minutes. The Thayer unit is defined as that quantity of material required to reduce the clotting time of the blood of 50% of a group of ten or more chicks, which

had been reared for the 14 days immediately following receipt from the hatchery on a diet practically devoid of vitamin K, to 10 minutes within the 3 days of administration of the test material. In a subsequent paper, Thayer et al. ('39 a) introduced dosage-response curves found to be of value only when several groups of birds were used, as it was necessary to find the dosage, the administration of which produced a response that fell on the curve, preferably near its midpoint. These investigators (Richert et al., '40) emphasized also the necessity of ascertaining the response of each group of chicks to a standard, believing that the variability of the degree of deficiency of different lots was the reason for gross inaccuracies in the bioassay.

Ansbacher's ('38) report that chicks responded to vitamin K within a very short time, which according to Cheney ('40) may be less than 30 minutes, caused Thayer et al. ('39 b) to reduce their period of assay to 18 hours and to administer the test material in a single dose. The Thayer-Doisy unit is based upon the shorter test and its definition is the same as that of the Thayer unit except for the assay period.

Dann ('39) adopted the longer technic of Thayer et al. ('39 a), and Macfie et al. ('39) further prolonged the assay period by giving the test material divided into four equal portions on 4 successive days and killing the animals on the fifth day, i.e., after a no-dose period of 24 hours, for the determination of clotting and prothrombin times. This method was used also by Almquist and Klose ('39 d) after they had found irregularity in the results obtained with preventive methods.

A rapid curative assay was advocated by Ansbacher ('39) whose unit is defined as the minimum amount necessary to render the blood clotting time of the vitamin K-deficient chick, weighing 70 to 100 gm., normal within 6 hours after administration. He recommended that assays be made with a constant amount of menstruum, namely 0.10 ml., since the oil used as a medium for administering the test material appeared to interfere to a small but noticeable degree with the potency, an observation recently confirmed by Thayer et al. ('40).

Furthermore, he preferred to make use of chicks showing pronounced hemorrhages in addition to markedly prolonged clotting times, thus assuring a more uniform deficiency. Indeed, Tidrick et al. ('39) found that hemorrhages appeared to occur only when the prothrombin content had fallen below about 10% of the values seen in normal chicks of the same age.

The use of a heated diet in place of an extracted fish meal ration employed by most investigators was recently proposed by Ansbacher ('40 c). One of the major sources of K-vitamin synthesis (Almquist and Stokstad, '35), so markedly interfering with bioassays, is thus eliminated, and the test period may be safely prolonged from 6 to 18 or 24 hours in order to allow adequate time for the maximum action of the vitamin (Tidrick et al., '39).

The ingenious bioassay of Flynn and Warner ('40) employs rats, the common bile duct of which is ligated after 7 to 8 weeks' subsistence on a diet known to be low in vitamin K. The prothrombin content of the blood, measured by the two-stage method of Warner et al. ('36) and Smith et al. ('37), was found to fall to a level of 10 to 25% of normal within 72 hours following the operation, and the response to treatment, either by the intraperitoneal or intravenous route, was determined by observing the speed and completeness of the prothrombin restoration. The rats can be employed several times, since the normal prothrombin level, brought about by vitamin K therapy, is maintained at best for a few days, but they become unsuitable as soon as general debility and hepatic cirrhosis occur as a result of bile duct ligation.

VITAMIN K UNITS

The approximate relationships of various vitamin K units were estimated by Dam ('40), by Brinkhous ('40), by Riegel ('40), and Ansbacher ('40b) analyzed the bioassay methods for the purpose of evaluating 1 unit in terms of any other one (table 1). Dam's standard spinach preparation (Dam and Glavind, '38), containing 500 Dam units per gram, was found to have a potency of 1 Ansbacher unit in 40 mg., i.e., 25 units/gm.

Therefore, 1 Ansbacher unit equals 20 Dam or 20 Schønheyder units, the two latter ones being of the same order of magnitude.

A concentrate from alfalfa (Fernholz et al., '39), having a potency of 1 Ansbacher unit in 50 μg., was assayed by the 3-day curative test of Thayer et al. ('39 a) and found to contain 1 Thayer unit in 50 μg. Therefore, Ansbacher's unit equals Thayer's unit, an expected result, since Dam et al. ('38) had noted "that the clotting power may be rendered normal between 4 and 6 hours after one single intravenous injection. The amount of vitamin injected was not very far from that which would have resulted in normal clotting power if it had been given on 3 successive days with 1/3 on each day."

TABLE 1

Units of vitamin K

1 Ansbacher unit equals	0.16 ml. of the Almquist Reference Standard	
	20 (1938) Dam	units
	12½ (1940) Dam-Glavind	
	½ (1938) Dann	
	1¼ (1939) Dann	
	20 (1936) Schønheyder	
	1 (1938) Thayer	
	2 (1939) Thayer-Doisy	

Thayer et al. ('39 b) noticed that the Thayer unit equals 2 Thayer-Doisy units as shown by their statement: "Actually, with birds of the same degree of deficiency approximately twice as much vitamin is required for a 50% response by the 3-day procedure" than by the 18-hour assay. Therefore, Ansbacher's unit should be equal to 2 Thayer-Doisy units. Indeed, Ansbacher et al. ('39, '40) found that Thayer-Doisy's 18-hour test required only one-half of the vitamin material needed in Ansbacher's 6-hour assay, and Tidrick et al. ('39) confirmed this observation in a quantitative study on prothrombin restoration in chicks.

The Dann unit, called "Almquist unit" by Elliot et al. ('40), Greer ('40), and Fitzgerald and Webster ('40), was claimed to equal 37½ Dam units (Greer, '40; Fitzgerald and Webster, '40). Calculating with 20 Dam units per Ansbacher unit, the latter should be equal to about ½ (i.e., 0.533) Dann unit. In the definition of Dann's unit by Snell ('39) reference is made

to "gram of a chick's body weight." If "gram" were correct, 100 Dann, i.e., 200 Ansbacher units would be required by a 100-gm. chick; however, 1 Ansbacher unit is therapeutically effective in a 100-gm. chick and prophylactically only 0.2 Ansbacher unit is needed (Ansbacher, '40 a). Thus the Dann unit refers to "kilogram of a chick's body weight."

TABLE 2

Potency of methylnaphthoquinone and phylloquinone

AUTHORS AND THEIR CO-WORKERS	INSTITUTION	METHYLNAPHTHOQUINONE ANSBACHER UNITS PER MILLIGRAM			PHYLLOQUINONE STANDARD UNITS[1] PER MILLIGRAM
Almquist	U. of California	3	30	1700	262–290
Ansbacher	Squibb Institute	2000	2000	2000	250 [2]
Dam	U. of Copenhagen			2000	500
Dann	Abbott Labs.			2000	300–400
Doisy	U. of St. Louis	5	500	2000	333–500
Kamm	Parke, Davis & Co.			2000	420–480
Moll	Merck Labs. (Germany)			3000	
Sampson	Merck Institute	< 2	850	1700	333
Sjögren	Astra Labs. (Sweden)			1700	500
		July 1939	Sept. 1939	More recently	

[1] Methylnaphthoquinone = 1000 units/mg. [2] Only 70 units/mg. in 6-hour test.

The Almquist et al. ('38) reference standard was found (Ansbacher, '40 a) to contain 1.56% of total solids, 2½ mg. of which had a potency of an Ansbacher unit. Therefore, the activity of this standard preparation is 1 Ansbacher unit in 0.16 ml. (0.10 ml. according to Almquist and Klose, '40).

VITAMIN K STANDARD UNIT

Proposals for an international standard vitamin K preparation appear in the current literature and the use of 2-methyl-1,4-naphthoquinone for this purpose was first suggested by Thayer et al. ('39 d). The results obtained with this substance in the various laboratories are a particularly striking illustration of the erroneous results so frequently occurring in vitamin K assays. In order to facilitate an interpretation of the published data, expressed in different units and reference standard equivalents, they were converted into Ansbacher units (table 2).

The antihemorrhagic activity of this methylnaphthoquinone was first mentioned in the July, 1939 issue of the Journal of the American Chemical Society where the following potency data appear: 3000 units per gram according to Almquist and Klose ('39 b); 5000 units per gram reported by Thayer et al. ('39 c) who stated that the activity of this compound is relatively insignificant when compared with the natural vitamin K_1 or K_2; 2,000,000 units per gram found by Ansbacher and Fernholz ('39) who emphasized that methylnaphthoquinone is practically as active as vitamin K. Two months later, Fernholz and Ansbacher ('39) confirmed their discovery and Thayer et al. ('39 d) published revised data showing: "The potency of 2-methyl-1,4-naphthoquinone (Thayer-Doisy units) agrees with the value previously assigned to the natural vitamin K_1, namely 1000 units per milligram. These results also confirm the findings of Ansbacher and Fernholz." Simultaneously, Almquist and Klose ('39 c) disclosed that methylnaphthoquinone had been reassayed and found to have a potency of 30,000 units per gram, and that "it is by no means as active as vitamin K." Later, however, Almquist and Klose ('39 d) described the "amazingly high activity of the compound" and fully confirmed the data of Ansbacher and Fernholz. Tishler and Sampson ('39) indicated that a potency of less than 2000 units per gram had been found in their initial tests with this substance and stated: "Following the appearance of the extremely interesting report of Ansbacher and Fernholz, we reinvestigated the activity of 2-methyl-1,4-naphthoquinone, and we are now in complete agreement with them." Further confirmatory publications appeared more recently from Dann ('39), Hepding and Moll ('39), Sjögren ('39), Dam et al. ('40 a), Emmett et al. ('40), and Thayer et al. ('40) with the result that this compound is now recognized as the most potent substance with vitamin K-activity (Riegel, '40).

The testing of methylnaphthoquinone, the vitamin K standard, in the various laboratories has resulted in the publication of new data on the relationships of various units. Dam et al. ('40 a) claimed it to have a potency of 25,000,000 Dam units

per gram, and because Ansbacher found 2,000,000 units, Ansbacher's unit equals 12½ Dam units. Since previously a ratio of 1:20 had been found for these 2 units, Dam ('40) suggested that the difference may be explained by the fact that Ansbacher's method was a rapid one in which the rate of absorption may lower the results. However, an analysis of Dam's data permits the conclusion that the difference in the ratio of 1:20 to that of 1:12½ is due to a change in Dam's unit. The potency of phylloquinone (vitamin K_1) was 20 Dam units per microgram in the earlier reports of Dam et al. ('39) and of Karrer and Geiger ('39), but it is now only 12½ Dam-Glavind units per microgram (Dam et al., '40 b).

Apparently Dann's unit has also been modified. Dann ('39) found methylnaphthoquinone to have a potency of 2,500,000 Dann units per gram; calculating with 25,000,000 Dam units per gram, the Dann unit corresponds to 10 Dam units. Previously, Dann's unit was claimed to equal 37½ Dam units; indeed, Dann ('39) observed that 75 mg. of Dam's standard with 500 Dam units per gram gave a response of a Dann unit, i.e., 13.3 Dann units per gram and a ratio of 1:37½ for the two units. In the same publication appeared the methylnaphthoquinone data from which the ratio of 1:10 was calculated. Therefore, Dann dealt with two different Dann units; namely, ½ of the one and 1¼ of the other one corresponded to Ansbacher's unit.

Following the suggestion of Thayer et al. ('39 d) to assign a value of 1 standard unit to 1 μg. of 2-methyl-1,4-naphthoquinone, several authors expressed the antihemorrhagic potency of phylloquinone in terms of this unit (table 2). However, Ewing et al. ('39) proposed 2-methyl-1,4-naphthohydroquinone diacetate as a standard, because it is less sensitive to light than methylnaphthoquinone. In view of the fact that the latter compound was found to have clinically the same prophylactic and therapeutic action as concentrates of naturally occurring vitamin K, Macfie et al. ('39) advocated to dispense altogether with assays and a standard preparation, especially since "biological tests cannot be depended on always to give clear-cut results."

In conclusion, it seems desirable to emphasize that the chemistry of vitamin K has been rapidly elaborated in spite of the difficulties inherent in bioassays. The clinical data show conclusively that this vitamin is essential for the formation and activation of prothrombin. Synthetic prothrombinogenic substances, such as, e.g., the standard methylnaphthoquinone, have now become part of the armamentarium of the surgeon and pediatrician. Consequently, many a jaundiced patient may be safely subjected to operation, since with the advent of vitamin K therapy, he is no longer such a serious surgical risk, and many a baby may be saved from hemorrhagic disease of the newborn.

LITERATURE CITED

ALMQUIST, H. J., AND A. A. KLOSE 1939 a Determination of the antihaemorrhagic vitamin. Biochem. J., vol. 33, p. 1055.

———— 1939 b The antihemorrhagic activity of certain naphthoquinones. J. Am. Chem. Soc., vol. 61, p. 1923.

———— 1939 c Synthetic and natural antihemorrhagic compounds. Ibid., vol. 61, p. 2557.

———— 1939 d Antihemorrhagic activity of 2-methyl-1,4-naphthoquinone. J. Biol. Chem., vol. 130, p. 787.

———— 1940 Comparative activities of certain antihemorrhagic compounds. Proc. Soc. Exp. Biol. and Med., vol. 45, p. 55.

ALMQUIST, H. J., E. MECCHI AND A. A. KLOSE 1938 Estimation of the antihaemorrhagic vitamin. Biochem. J., vol. 32, p. 1897.

ALMQUIST, H. J., AND E. L. R. STOKSTAD 1935 Hemorrhagic chick disease of dietary origin. J. Biol. Chem., vol. 111, p. 105.

———— 1937 a The gizzard factor of the chick. J. Nutrition, vol. 13, p. 339.

———— 1937 b Assay procedure for vitamin K (anti-hemorrhagic vitamin). Ibid., vol. 14, p. 235.

ANSBACHER, S. 1938 New observations on the vitamin K deficiency of the chick. Science, vol. 88, p. 221.

———— 1939 A quantitative biological assay of vitamin K. J. Nutrition, vol. 17, p. 303.

———— 1940 a Unpublished data.

———— 1940 b Vitamin K. Proc., Am. Soc. Biol. Chem., J. Biol. Chem., vol. 133, p. iii.

———— 1940 c A new type of vitamin K-deficient diets. Proc. Soc. Exp. Biol. and Med., vol. 44, p. 248.

ANSBACHER, S., AND E. FERNHOLZ 1939 Simple compounds with vitamin K activity. J. Am. Chem. Soc., vol. 61, p. 1924.

ANSBACHER, S., E. FERNHOLZ AND M. A. DOLLIVER 1940 Vitamin K-active derivatives of 2-methyl-1,4-naphthohydroquinone. Ibid., vol. 62, p. 155.

ANSBACHER, S., E. FERNHOLZ AND H. B. MACPHILLAMY 1939 Natural vitamin K and synthetic vitamin K_1. Proc. Soc. Exp. Biol. and Med., vol. 42, p. 655.

BRINKHOUS, K. M. 1940 Plasma prothrombin; vitamin K. Medicine, vol. 19, p. 329.

CHENEY, G. 1939 The intramuscular injection of vitamin K. J. Lab. Clin. Med., vol. 24, p. 919.

———— 1940 The plasma coagulation time as a simple test for vitamin K deficiency. Am. J. Med. Sci., vol. 200, p. 327.

DAM, H. 1935 The antihaemorrhagic vitamin of the chick. Biochem. J., vol. 29, p. 1273.

———— 1940 Fat-soluble vitamins. Ann. Rev. Biochem., vol. 9, p. 353.

DAM, H., A. GEIGER, J. GLAVIND, P. KARRER, W. KARRER, E. ROTHSCHILD AND H. SOLOMON 1939 Isolierung des Vitamins K in hochgereinigter Form. Helv. Chim. Acta, vol. 22, p. 310.

DAM, H., AND J. GLAVIND 1938 Determination of vitamin K by the curative blood-clotting method. Biochem. J., vol. 32, p. 1018.

———— 1940 Determination of prothrombin. J. Am. Med. Assoc., vol. 115, p. 149.

DAM, H., J. GLAVIND AND P. KARRER 1940 a Die biologische Aktivität der natürlichen K-Vitamine und einiger verwandter Verbindungen. Helv. Chim. Acta, vol. 23, p. 224.

DAM, H., J. GLAVIND, L. LEWIS AND E. TAGE-HANSEN 1938 Studies on the mode of action of vitamin K. Skand. Arch. Physiol., vol. 79, p. 121.

DAM, H., J. GLAVIND AND N. NIELSEN 1940 b Weitere Untersuchungen über die Bildung und Bedeutung des Vitamin K im Pflanzenorganismus. Z. physiol. Chem., vol. 265, p. 80.

DANN, F. P. 1938 Vitamin K assays. Proc., Am. Physiol. Soc., Am. J. Physiol., vol. 123, p. 48.

———— 1939 Quantitative biological assay of vitamin K and its application to several quinone compounds. Proc. Soc. Exp. Biol. and Med., vol. 42, p. 663.

ELLIOT, M. C., B. ISAACS AND A. C. IVY 1940 Production of "prothrombin deficiency" and response to vitamins A, D and K. Proc. Soc. Exp. Biol. and Med., vol. 43, p. 240.

EMMETT, A. D., R. A. BROWN AND O. KAMM 1940 Comparison of the antihemorrhagic activity of natural and synthetic vitamin K_1 with the proposed standard 2-methyl-1,4-naphthoquinone. J. Biol. Chem., vol. 132, p. 467.

EWING, D. T., J. M. VANDENBELT AND O. KAMM 1939 The ultraviolet absorption of vitamins K_1, K_2, and some related compounds. J. Biol. Chem., vol. 131, p. 345.

FERNHOLZ, E., AND S. ANSBACHER 1939 Vitamin K activity of synthetic phthiocol. Science, vol. 90, p. 215.

FERNHOLZ, E., S. ANSBACHER AND M. L. MOORE 1939 On the color reaction for vitamin K. J. Am. Chem. Soc., vol. 61, p. 1613.

FITZGERALD, J. E., AND A. WEBSTER 1940 Effect of vitamin K administered to patients in labor. Am. J. Obstet. Gynec., vol. 40, p. 413.

FLYNN, J. E., AND E. D. WARNER 1940 Prothrombin levels and synthetic vitamin K in obstructive jaundice in rats. Proc. Soc. Exp. Biol. and Med., vol. 43, p. 190.

GREER, A. E. 1940 The place of vitamin K in hemorrhagic diseases. Texas State J. Med., vol. 36, p. 218.

HEPDING, L., AND T. MOLL 1939 Über das Vitamin K. Die Methoden der biologischen Prüfung sowie Auswertung K-wirksamer Stoffe. E. Merck's Jahresber., vol. 53, p. 5.

KARRER, P., AND A. GEIGER 1939 Vitamin K aus Alfalfa. Helv. Chim. Acta, vol. 22, p. 945.

MACFIE, J. M., A. L. BACHARACH AND M. R. A. CHANCE 1939 The vitamin K activity of 2-methyl-1,4-naphthoquinone and its clinical use in obstructive jaundice. Brit. Med. J. of Dec. 23, p. 1220.

QUICK, A. J. 1938 The nature of the bleeding in jaundice. J. Am. Med. Assoc., vol. 110, p. 1658.

RICHERT, D., S. A. THAYER, R. W. MCKEE, S. B. BINKLEY AND E. A. DOISY 1940 Bioassay of water-soluble antihemorrhagic compounds by intravenous administration. Proc. Soc. Exp. Biol. and Med., vol. 44, p. 601.

RIEGEL, B. 1940 Vitamins K. Erg. Physiol., biol. Chem., exp. Pharmak., vol. 43, p. 133.

SCHØNHEYDER, F. 1936 a Nogle Undersøgelser over den biologiske Virkning og Standardiseringen af det antihaemorrhagiske Vitamin K. Ph.D. Thesis, University of Copenhagen, p. 17.

———— 1936 b The quantitative determination of vitamin K. Biochem. J., vol. 30, p. 890.

SJÖGREN, B. 1939 Die Vitamin K-Wirkung einiger Naphthochinone und Naphthochinonderivate. Z. physiol. Chem., vol. 262, p. 1.

SMITH, H. P., E. D. WARNER AND K. M. BRINKHOUS 1937 Prothrombin deficiency and the bleeding tendency in liver injury (chloroform intoxication). J. Exp. Med., vol. 66, p. 801.

SNELL, A. M. 1939 Vitamin K: its properties, distribution and clinical importance. J. Am. Med. Assoc., vol. 112, p. 1457.

THAYER, S. A., S. B. BINKLEY, D. W. MACCORQUODALE, E. A. DOISY, A. D. EMMETT, R. A. BROWN AND O. D. BIRD 1939 d Vitamin K potencies of synthetic compounds. J. Am. Chem. Soc., vol. 61, p. 2563.

THAYER, S. A., L. C. CHENEY, S. B. BINKLEY, D. W. MACCORQUODALE AND E. A. DOISY 1939 c Vitamin K activity of some quinones. Ibid., vol. 61, p. 1932.

THAYER, S. A., D. W. MACCORQUODALE, R. W. MCKEE AND E. A. DOISY 1938 The biological assay of the antihemorrhagic factor (vitamin K). Proc., Am. Soc. Biol. Chem., J. Biol. Chem., vol. 123, p. CXX.

THAYER, S. A., R. W. MCKEE, S. B. BINKLEY AND E. A. DOISY 1940 Potencies of vitamin K_1 and of 2-methyl-1,4-naphthoquinone. Proc. Soc. Exp. Biol. and Med., vol. 44, p. 585.

THAYER, S. A., R. W. MCKEE, S. B. BINKLEY, D. W. MACCORQUODALE AND E. A. DOISY 1939 a Assay of vitamin K concentrates. Ibid., vol. 40, p. 478.

———— 1939 b The assay of vitamins K_1 and K_2. Ibid., vol. 41, p. 194.

THAYER, S. A., R. W. MCKEE, D. W. MACCORQUODALE AND E. A. DOISY 1937 Recovery from the anemia caused by a diet deficient in vitamin K. Ibid., vol. 37, p. 417.

TIDRICK, R. T., F. T. JOYCE AND H. P. SMITH 1939 Vitamin K deficiency and prothrombin levels. Effect of vitamin K administration. Ibid., vol. 42, p. 853.

TISHLER, M., AND W. L. SAMPSON 1939 Antihemorrhagic activity of simple compounds. J. Am. Chem. Soc., vol. 61, p. 2563.

WARNER, E. D., K. M. BRINKHOUS AND H. P. SMITH 1936 A quantitative study on blood clotting: prothrombin fluctuations under experimental conditions. Am. J. Physiol., vol. 114, p. 667.

THE EFFECT OF THE QUANTITY OF BASAL FOOD INTAKE UPON THE UTILIZATION OF VITAMIN A [1]

KATHLEEN DIETRICH MUELDER [2] AND EUNICE KELLY
Department of Foods and Nutrition, Division of Home Economics, Michigan State College, East Lansing

(Received for publication August 28, 1940)

In recent years there has developed a growing recognition of the importance of interrelations which undoubtedly exist among the various essential nutrients in a diet. The effect of individual constituents of a diet, for instance, on the utilization of vitamin A is a question which presented itself when the varying results obtained during the course of standardized biological assays for vitamin A were scrutinized. Numerous explanations of the causes of these variations in the response of rats under controlled conditions have been offered. Homogeneity of the colony from which the test animals have been selected, percentages of fat in the basal diet, and the quantity of basal ration consumed during the depletion period are factors named by Nelson and Swanson ('31) as affecting vitamin A determinations. Honeywell et al. ('31) reported that variations in yeast as a source of the B complex and the possibility of traces of vitamin A from yeast caused differences in the response of animals during a vitamin A assay. It has been suggested further by Javillier and Emerique ('31) that an adequate supply of vitamin D and the criteria for judging the state of depletion are important factors in the biological estimation of vitamin A.

[1] Approved by the director of the Michigan Agricultural Experimental Station for publication as Journal Article no. 457, n.s.

[2] Formerly Assistant Professor of Foods and Nutrition and Research Assistant in Foods and Nutrition at Michigan State College, East Lansing, Michigan.

Such observations by investigators carrying out the accepted technique for vitamin A determinations, as well as a general interest in the possible physiological interrelationships of dietary factors, suggested this study of the influence of different constituents in the basal ration upon the utilization of vitamin A. The problem was undertaken not from the point of view of its relation to the technique for assays but rather from the standpoint of its relation to vitamin A metabolism. The first phase of this experiment was planned to test the influence of the caloric intake from basal ration consumed during the test period upon the utilization of vitamin A, using rats with the same nutritional background and with similar inheritance. Rate of growth and incidence of "abscessed" areas were the criteria used to measure the response, to graduated levels of vitamin A, of animals consuming restricted, as compared with unrestricted, quantities of the basal ration. The data include the responses of 237 animals and were collected over a period of 2 years, 1935–1937.

PROCEDURE

The basal vitamin-A-deficient ration employed was essentially that recommended for assay according to the U. S. Pharmacopeia, XI Revision ('35). In order, however, to insure a sufficiency of the B vitamins at the restricted levels of food intake, the dried bakers' yeast was not incorporated into the ration, but a 0.5 gm. portion was fed daily as a separate supplement. Triads of animals (Haag, '34; Smith, '34) selected on a basis of age, weight, sex, and litter, were depleted on this ration.[3] A uniformity in weight range in a triad during the depletion period was maintained by controlling the food intake within the group. At the end of the depletion period one animal from each triad was selected as a negative control, one as a calorie control, and one as a positive control.

Since the food consumption of the calorie control rats was determined by that of the negative control animals, a possible

[3] Composition of basal ration: 18% vitamin-A-extracted casein, 4% Osborne and Mendel salt mixture, 73% irradiated corn starch, and 5% fat.

means of prolonging the survival time of the latter was sought in order to insure greater accuracy in the paired-feeding. Sherman and Storms ('25) demonstrated that an increase in age and in weight of the animals at the time of starting on a vitamin A deficient ration will cause a relatively small increase in depletion time but also a longer time of survival after depletion. These findings were used as a guide in determining the age and weight for starting the animals on experiment. The rats, weaned at an age of 25 to 28 days and having a weight of 50 to 55 gm., were continued on the stock ration [4] for approximately 2 weeks. In actual practice, the animals were started on the vitamin-A-deficient basal ration at an age of 39 to 43 days. The range in weight was 105 to 115 gm. for the males and 90 to 100 gm. for the females.

Body weight and food consumption were recorded weekly for the first 3 weeks, and twice a week for the remainder of the depletion period. Examinations for signs of ophthalmia were made at the same times. The criteria for deciding when a triad reached depletion were failure to gain in weight for 1 week and evidence of ophthalmia.

Oil dilutions [5] of reference cod liver oil (U. S. Pharmacopeia, XI) were prepared each week quantitatively, so that when fed from calibrated droppers, 2 drops of solution carried 1, 3 or 6 U. S. P. XI Units of vitamin A. This supplement was fed daily, the negative control animals being fed 2 drops of refined cottonseed oil. The positive and negative control animals were allowed the basal ration ad libitum during the experimental period. The calorie control rats were restricted to an allowance of basal ration equal to that consumed by the negative controls. The calorie control rat, however, in any one triad, was fed the same amount of the cod liver oil supplement (1, 3 or 6 International Units of vitamin A) as

[4] Composition of stock ration in grams: whole milk powder 300, whole wheat 500, oats 500, whole yellow corn 480, flaxseed oil meal 200, calcium carbonate 20, Kelco meal (containing seaweed) 80. On alternate days each animal receives 2 to 3 gm. raw lean beef and 2 to 3 gm. raw leaf lettuce.

[5] Refined cottonseed oil was used for this purpose as well as to furnish the 5% fat in the basal ration.

was given the positive control rat of the same triad. Because of the number of animals used in this experiment and the fact that the tests were run over a 2-year period, the supply of reference cod liver oil had to be replenished several times. Variations in vitamin A content of the different reference samples should not, in this study, modify the interpretation of results, because comparisons are made between groups of animals in which the calorie control and positive control from the same triad were always fed the cod liver oil supplement from the same dilution.

During the 4-week experimental period the animals were weighed weekly. The food consumption records for the positive controls were also made weekly; but daily weighings of the food consumed by the negative controls were recorded because these quantities were used as the basis for feeding the calorie controls. The average survival time of all negative controls of the triads was 21.2 days, 21.0 days and 22.6 days respectively for the 1-, 3- and 6-unit triads with a range in survival time of 13 to 28 days. In those triads in which the negative control animal died before the twenty-eighth day, food allowance for the calorie control was determined by the average food consumption of negative animals of other triads in corresponding periods of the experiment.

Complete gross autopsy findings were recorded, altl ough it was found that, for the purposes of comparison desired in this study, an enumeration of the number and location of proliferated areas of keratinized epithelial tissues furnished a useful, objective indication of the extent of vitamin A deficiency. Vaginal smears were made thrice weekly during the entire experiment. These data it was hoped would give an additional basis for interpretation of the findings, since the vaginal epithelium has been found to be sensitive to a vitamin A deficiency (Aberle, '33; Mason and Ellison, '35). It was found, however, that this test was too sensitive for the purpose of this work. Recovery from persistent keratinization, for instance, was too rapid to serve as a basis for differentiating the response to the same level of vitamin A supplement of

rats on restricted versus unrestricted intake of the basal ration.

DISCUSSION OF RESULTS

From table 1, in which are given summarized data (arithmetic means) for the depletion period, it may be seen that the manner of selecting triads of animals and the method of maintaining weight uniformity within triads during the depletion period resulted in uniform groups of animals at the beginning of the test period.

TABLE 1

Average ages, weights, and food consumptions of animals from weaning to end of depletion period on the vitamin A deficient diet

GROUP [1]	YEAR	TRIADS		WEIGHT AT WEANING [2]	DEPLETION PERIOD				
					Time	Age at beginning	Weight at		Weekly food consumption
		Male	Female				Beginning	Ending	
		No.	*No.*	*gm.*	*weeks*	*days*	*gm.*	*gm.*	*gm.*
1	1935–36	6	7	54.5	7.6	41.8	103.9	174.8	67.8
2	1936–37	7	7	58.4	8.3	39.9	103.2	180.7	76.4
3	1935–36	6	7	55.7	8.2	41.5	102.3	177.3	67.7
4	1936–37	6	4	60.4	8.2	40.2	107.1	189.0	78.5
5	1935–36	6	6	54.1	8.0	41.6	102.1	177.6	67.7
6	1936–37	6	5	57.9	7.8	40.5	102.9	176.8	75.6

[1] Groups 1 and 2 were put on supplements of 1 I.U. of vitamin A; groups 3 and 4 on 3 I.U., and groups 5 and 6 on 6 I.U.

[2] Average age at weaning was 27.9 days.

Since negative and calorie control rats of a given triad consumed the same quantity of basal diet but the negative control rat was denied vitamin A supplement, the differences in response of these animals was a direct measure of the effect of the vitamin A fed. Also, since calorie and positive control rats in a triad received the same level of vitamin A supplement but consumed different quantities of basal diet, differences in response of these animals measured the response due to variations in food intake.

Effect upon growth. In table 2 are given the summarized data for the 4-week experimental period for both sexes combined for the 2 years. A statistical analysis of weekly food

consumptions, weight changes, and number of "abscesses" evident at autopsy was calculated for each year and each sex, separately as well as combined, in order to determine if there was consistency in response during the 2 years. Since the

TABLE 2

Average weekly food intakes and weight changes during 4-week experimental period and "abscesses" evident at autopsy

YEAR AND DESCRIPTION OF ANIMALS	ANIMALS	LEVEL OF VITAMIN A	AVERAGE WEEKLY FOOD INTAKE [1]	AVERAGE WEEKLY WEIGHT CHANGE [1]	"ABSCESSES" EVIDENT AT AUTOPSY [2]
	No.	*Units* [3]	*gm.*	*gm.*	*no.*
1935–37 males and females					
Negative	27	0	41.0 ± 2.01	—12.2 ± 1.49	3.2 ± 0.25
Calorie	27	1	42.4 ± 1.44	— 8.3 ± 1.02	1.3 ± 0.26
Positive	25	1	53.6 ± 1.37	— 2.8 ± 1.11	1.7 ± 0.27
1935–37 males and females					
Negative	23	0	41.9 ± 2.38	—14.3 ± 1.67	3.5 ± 0.30
Calorie	23	3	43.9 ± 1.52	— 7.3 ± 1.09	1.3 ± 0.19
Positive	22	3	65.6 ± 1.33	+ 5.6 ± 0.63	1.0 ± 0.22
1935–37 males and females					
Negative	23	0	36.9 ± 2.43	—16.3 ± 1.61	3.7 ± 0.25
Calorie	23	6	40.8 ± 1.52	— 8.1 ± 0.98	1.4 ± 0.21
Positive	23	6	70.8 ± 1.67	+ 8.0 ± 0.68	0.9 ± 0.19

[1] Arithmetic mean ± standard error.

[2] Areas that most frequently showed "abscesses" were middle ear, base of tongue, lymph glands in neck, salivary glands, and genito-urinary tract.

[3] International Units.

2 years' response was similar and, for the purpose of discussion, the mean response of the combined groups will be considered, only the summarized data for the combined groups are presented.

There was increasing difficulty at each higher level of vitamin A intake in keeping the calorie control group adjusted to the low level of food consumed by the negative control group. A slight increase in caloric intake at each increase in level of vitamin A might be expected because of increased appetite

and improved general well-being in the calorie control rats receiving some vitamin A. Furthermore, failure of the negative control rats to consume the basal diet during the last days of survival caused some of the differences observed.

In table 3 are given the average weekly differences in response of all seventy-three calorie control animals as compared with all negative control animals. During the 4-week experimental period, the twenty-seven calorie control rats given 1 unit of vitamin A lost 3.9 gm. less per week than did the corresponding twenty-seven negative control rats. This decreased loss, which, therefore, might be considered a "gain" of 3.9 gm. over that of the negative control animals accompanied an increase in caloric intake of but 5.7 calories from

TABLE 3

Comparison of weight change and food intake in negative and calorie control animals

VITAMIN A LEVEL	NUMBER OF TRIADS	DIFFERENCE IN			EXPECTED GAIN IN WEIGT DUE TO DIFFERENCE IN CALORIE INTAKE
		Weight change per week	Food intake per week	Calorie intake per week	
I.U.[1]		*gm.*	*gm.*	*Cal.*	*gm.*
1	27	3.9	1.4	5.7	+0.14
3	23	7.0	2.0	8.18	+0.21
6	23	8.2	3.9	15.95	+0.40

[1] International Unit.

1.4 gm. of food. This increase of food would not have been expected to give more than 0.14 gm. gain in weight per week, if calculated on the basis of grams gained per total calories ingested. This difference in weight of 3.9 gm. per week for the 4-week period, then, may be considered a measure of the response to the 1 unit of vitamin A administered. There was an increase of 3.1 gm. gain at the 3-unit level over that of the 1-unit level, and 1.2 gm. gain at the 6-unit level over that of the 3-unit level which was greater than can be accounted for by the slight increase in food intake. This observed "leveling off" of weight increases would indicate that the 3 and 6 unit levels of vitamin A were beyond the most sensitive areas of a curve of response of the animals used in these experiments.

In table 4 are given the differences in average weekly gain in weight of all positive control rats over that of the calorie control rats of corresponding triads. Since the calorie control rats and positive control rats were receiving identical doses of vitamin A supplement (1, 3 or 6 units), in any one group, but consuming different quantities of food, differences in average gain in weight between the two groups of animals were to be explained on the basis of differences in food intakes. There was an increased gain in weight which was directly related to the increased caloric intake at each succeedingly

TABLE 4

Comparison of weight change and food intake in caloric and positive control animals

VITAMIN A LEVEL	NUMBER OF TRIADS	DIFFERENCE IN			RATIO OF DIFFERENCE IN WEIGHT CHANGE TO CALORIE INTAKE	GAIN IN WEIGHT PER CALORIE [2]
		Weight change per week	Food intake per week	Calorie intake per week		
I.U. [1]		*gm.*	*gm.*	*cal.*		*gm.*
1	27	5.5	11.2	45.8	0.120	N—0.073 C—0.048 P—0.013
3	23	12.9	21.7	88.75	0.145	N—0.083 C—0.036 P+0.021
6	23	16.1	30.0	122.7	0.131	N—0.108 C—0.048 P+0.028

[1] International Unit.

[2] N = negative control animal; C = calorie control animal; P = positive control animal.

higher level of vitamin A. The ratio of the weight increase to the calorie increase (column 6, table 4) however, was strikingly similar for each level of vitamin intake. It would seem that there was improvement in general well-being and perhaps even in appetite (Sampson, Dennison and Korenchevsky, '32) which induced increased caloric intakes and gains in weight with increased levels of vitamin A.

Nelson and Swanson ('31) have reported that the quantity of basal ration consumed during the depletion period is the measured variable having the greatest percentage effect on

weight gains in a vitamin A assay. In this experiment the food intake of each triad was adjusted during the depletion period so as to keep each member of the triad gaining at a uniform rate and thus have an even group at the end of the depletion period. It will be seen (table 4) that during the experimental period, also, the caloric intake was an important factor in producing weight changes, the relative proportion of weight increase differing at different levels of vitamin intake. Expressed as percentage, 59% of the gain in weight of the positive control group over that of the negative control group was due to increased calories at the 1-unit level, 64% at the 3-unit level, and 66% at the 6-unit level of vitamin intake. A part of the discrepancies in the literature in respect to weight responses with vitamin A assays may be due to differences in food intake. It has been calculated from these data that a gain of 0.025 gm. per week for each calorie ingested might be expected in the response of animals evenly balanced as to sex on this experimental diet.

Although not indicated in the tables, males demonstrated a slightly higher gain in body weight per calorie of food ingested than did females. This is in agreement with the findings of Palmer and Kennedy ('31) on sex differences in gain in weight and illustrates the necessity of having the groups evenly matched in respect to sex when using weight as a criterion of differences in response.

All of these data have been tested by the "t" tests for significance of differences in mean response according to the formula $t = \frac{M - M_1}{\sqrt{\frac{(\sigma)^2}{N} + \frac{(\sigma_1)^2}{N_1}}}$, and were found to be highly significant. That is, the "t" values for the mean differences in response between the negative and calorie and between the calorie and positive groups were high.

Effect on incidence of pus-like accumulations in organs and tissues (*abscesses*). Another criterion for judging vitamin A utilization was the presence or absence of areas of pus-like material in organs, glands, and tissues as examined grossly at autopsy. It is recognized that, if these areas were typical

of vitamin A deficiency, they probably were in most instances accumulations of keratinized epithelial cells (Wolbach, '37). For convenience, these pus-like accumulations have been tabulated as "abscesses" evident at autopsy. It is seen that the negative control animals at each level of vitamin administration presented essentially the same picture in respect to number of "abscesses," namely, 3.2 for the 1-unit group, 3.5 for the 3-unit group, and 3.7 for the 6-unit group, showing the uniformity of negative groups in developing "abscesses" when deprived of vitamin A on this experimental diet.

Administration of even 1 International Unit of A reduced the number of areas of epithelial proliferation at least 50%.

The use of 6 units of vitamin A did not result in any significant improvement over 1 unit of vitamin A in preventing occurrence of "abscesses" seen at autopsy. However, the general appearance of the 6-unit group was better than that of the 1-unit group. At the end of the 4-week experimental period, there was no external evidence of ophthalmia among the group receiving 6 units of vitamin A. It is believed by Coward ('38) that four or five times as high levels of vitamin A intake are necessary to obtain a removal of all evidence of ophthalmia as are required to stimulate gain in weight. It is possible, in the light of the results of this study, that the administration of even more than five times the quantity of vitamin A required to produce weight gains is necessary to prevent all evidence of ophthalmia. While results from these experiments indicate a definite reduction of "abscesses" by the administration of vitamin A, analyses of separate groupings of animals indicate considerable variation in response at different levels of supplement, between sexes, and between the 2 years of the study. This conclusion concerning the effect of vitamin A intake upon incidence of "abscesses" has been substantiated by the results of the "t" tests for significance of difference in mean response. In all cases the "t" values for the mean difference in response between the negative and calorie control animals or between the negative and positive control animals were high, indicating

a highly significant reduction in "abscesses" by administration of the vitamin.

The "t" values for the mean difference in response due to calories ingested between the calorie and positive control animals were small, showing that the caloric intake had no statistically significant effect upon the incidence of "abscesses." However, it will be noted that with the calorie control groups there was no reduction in incidence of "abscesses" by the administration of 6 units of vitamin A as compared with 1 unit of vitamin A (1.4 and 1.3 "abscesses" respectively), while with the positive control groups and similar increases in vitamin A there was a reduction in number of such areas from 1.7 to 0.9. Although this difference in incidence of "abscesses" reached only the borderline of significance (there was a "t" value of 2.29 whereas a "t" value of 2.67 would have been required for statistical significance) there was some indication of improved metabolism with an unrestricted when contrasted with a restricted food intake when the higher levels of vitamin A were administered.

SUMMARY

The influence of the quantity of basal food intake on vitamin A utilization has been studied by means of carefully controlled triads of rats as measured by rate of growth and incidence of "abscessed" areas. For the levels of vitamin A used in this experiment, the caloric intake was responsible for a greater proportion of the gain in weight during a 4-week period than was the unitage of vitamin A administered, the percentage of the gain in weight from caloric intake increasing at each elevation of vitamin intake. When the intake of vitamin A was identical in different groups of animals, the gain in weight was directly related to the quantity of basal diet consumed during the period of observation.

There was a highly significant reduction in number of "abscessed" areas by the administration of either 1, 3 or 6 International Units of vitamin A below the number of such areas exhibited by the negative groups, but no statistically

significant difference between the levels of vitamin used in this experiment. The quantity of basal food intake showed no statistically significant influence upon incidence of "abscessed" areas. However, there was some indication of an advantage of unrestricted as compared with restricted food intake at the highest level (6 units) of vitamin A intake.

Acknowledgment is gratefully made to Dr. Thelma Porter for her interest and advice in the preparation of the manuscript and to Dr. Marie Dye, Dr. Jean Hawks, and Miss Flora Hanning for suggestions in the planning of the problem.

LITERATURE CITED

ABERLE, S. B. D. 1933 Continual cornified vaginal cells as an index of avitaminosis in rats. J. Nutrition, vol. 6, p. 1.

COWARD, K. H. 1938 The Biological Standardization of the Vitamins, London: Bailliere, Tindall, and Cox.

HAAG, J. R. 1934 An adaptation of the paired-feeding method for the determination of the supplementary value of proteins. J. Nutrition, vol. 8, p. 235.

HONEYWELL, HANNAH E., ADAMS R. DUTCHER AND JACOB O. ELY 1931 Vitamin studies. XVIII. The biological assay of food materials for vitamin A as influenced by yeast from various sources. J. Nutrition, vol. 3, p. 491.

JAVILLIER, M., AND L. EMERIQUE 1931 Physiological measurement of vitamin A activity. Bull. Soc. Chem. Biol., vol. 13, p. 771.

MASON, K. E., AND E. T. ELLISON 1935 Changes in the vaginal epithelium of the rat after vitamin A deficiency. J. Nutrition, vol. 9, p. 735.

NELSON, P. MABEL, AND PEARL P. SWANSON 1931 The influence of experimental technic during the preliminary period in vitamin A determinations. Iowa Agr., Expt. Sta. Ann. Rept., p. 90.

PALMER, L. S., AND CORNELIA KENNEDY 1931 The fundamental food requirements for the growth of the rat. VI. The influence of the food consumption and the efficiency quotient of the animal. J. Biol. Chem., vol. 90, p. 545.

SAMPSON, M. M., M. DENNISON AND V. KORENCHEVSKY 1932 CLVII. The absorption of nitrogen and of fat from alimentary canal of rats kept on a vitamin A-deficient diet. Biochem. J., vol. 26, p. 1315.

SHERMAN, H. C., AND L. B. STORMS 1925 The bodily store of vitamin A as influenced by age and other conditions. J. Am. Chem. Soc., vol. 47, p. 1653.

SMITH, MARGARET E. 1934 The effect of vitamin A deficiency on the concentration of the blood lipids of albino rats. J. Nutrition, vol. 8, p. 675.

U. S. PHARMACOPOEIA, XI REVISION 1935 P. 478.

WOLBACH, S. B. 1937 The pathologic changes resulting from vitamin deficiency. J. Am. Med. Assoc., vol. 108, p. 7.

THE HEAT PRODUCTION AND BLOOD AND URINE CONSTITUENTS AFTER ADMINISTRATION OF l (—) HISTIDINE TO THE DOG [1]

A. G. EATON AND J. R. DOTY
Department of Physiology, Louisiana State University, School of Medicine, New Orleans

(Received for publication August 6, 1940)

Standard textbooks and reviews generally credit histidine with no specific dynamic action. The basis for these statements appears to be a single experiment performed by Rapport and Beard ('28) in which they administered 5 gm. of histidine dihydrochloride by stomach tube to a dog and observed no increase in heat production during the next 3 hours. If one considers that approximately one-third of the 5 gm. administered was hydrochloric acid, it is easy to see that a small rise in heat production, following the feeding of so small an amount of histidine, might not be apparent. Furthermore, the dihydrochloride of histidine is a very acid substance and might easily produce conditions within the gastro-intestinal tract which might delay absorption. Nor has the effect of the administration of this quantity of hydrochloric acid upon the heat production been adequately evaluated. Thus, the effect of a small amount of histidine, absorbed over a prolonged period, might not be observable.

Reinwein ('28) was unable to obtain increases in the oxygen consumption of rats, guinea pigs and liver slices following histidine administration but upon the administration of a single dose of 25 gm. of histidine to a 13.5 kg. dog did observe

[1] Aided by a grant from the Committee on Scientific Research, American Medical Association. A preliminary report of these studies was presented before the fifty-second meeting of the American Physiological Society at New Orleans, March 13, 1940.

an average increase of 19.9% in the heat production. He calculated the increase to be 7.2 Cal. per gram of administered nitrogen. However, since he gave no data regarding either the excretion of the compound or the quantity of urea formed from it, we are unable to make a direct comparison of his results with ours.

Not only were we interested in arriving at an accurate measure of the specific dynamic action of histidine but we wished to determine the rate of excretion, deamination and urea formation following its intravenous injection. It was hoped that these data together with previously published figures for glycine, lysine and arginine (Doty and Eaton, '39; Eaton and Doty, '39) might throw some light on the theory of urea formation of Krebs and Henseleit ('32) as applied to the intact normal dog.

METHODS

With but few exceptions the plan of experiments and the methods of analysis have already been described (Doty and Eaton, '39). Urinary amino nitrogen was determined by the manometric method of Van Slyke ('29) upon the silver histidine precipitate prepared according to Block ('34).

It was planned to give histidine monohydrochloride in amounts practically equal to the quantities of arginine monohydrochloride and lysine monohydrochloride administered in former experiments so that a fair basis of comparison might be obtained. However, a preliminary trial showed the monohydrochloride of histidine to be unsuitable for satisfactory intravenous injection. In one experiment reported in this paper, the monohydrochloride (9.6 gm.) was carefully neutralized to pH 7.0 (glass electrode) by means of sodium hydroxide and the resulting solution injected intravenously. Since we wished, however, to avoid possible complications resulting from the injection of a comparatively large quantity of sodium chloride we resorted to the use of the l(—)histidine (free base). The four remaining experiments included in the tables were performed by dissolving the histidine (free base) in

approximately 110 cc. of physiological saline, sterilizing by heat, and injecting the resulting solution into a saphenous vein at a constant rate during a period of 10 minutes. Because restlessness during several attempted experiments had prevented us from following the heat production accurately, we performed the two experiments on dog 6 H and the one on dog 7 H under deep and constant sodium amytal anesthesia in a room with the temperature sufficiently high to maintain the dog's temperature at the normal level.

Preparation of free histidine base. This compound was readily prepared from a saturated aqueous solution of the monohydrochloride by slowly adding an amount of a concentrated aqueous solution of lithium hydroxide exactly equivalent to the hydrogen chloride. Precipitation of the histidine began almost immediately and was rendered more complete by adding an equal volume of 95% ethyl alcohol and by cooling the mixture overnight in a refrigerator at 0°C. The crystals were collected on a glass filter, freed of the mother liquor by suction and thoroughly washed with 95% alcohol. Nearly theoretical yields of histidine were obtained. When necessary, the last traces of lithium chloride can be removed by solution of the histidine in the minimum amount of hot water and by reprecipitation as above with 95% alcohol.

RESULTS

The results of blood analyses, as well as average comparative values for intravenous experiments with glycine, lysine, and arginine, compiled from previously published papers (Doty and Eaton, '39; Eaton and Doty, '39), are shown, for rapid comparison, in table 1. Figures for heat production and urinary nitrogen fractions are included in table 2.

The blood amino nitrogen changes after histidine administration tend to parallel those obtained when arginine was injected, except that the initial decrease is somewhat faster for arginine. At least a partial explanation for this difference is obvious when one examines table 2. The average excretion of histidine is only 1.44 gm. in contrast to 2.92 gm. for arginine.

Since the fastest rate of excretion occurs when the concentration in the blood is at a high level, it is very probable that the initial rate of excretion of arginine is about twice that of histidine. The fall in blood amino N after lysine is due almost entirely to excretion of that compound into the urine, while

TABLE 1

Blood amino nitrogen and blood urea nitrogen values after intravenous administration of histidine

DOG NO.	ADMINISTERED GRAMS	MILLIGRAMS PER 100 CC. OF BLOOD						
		Control period (hours before administration)		Experimental period (hours after administration)				
		1	0.5	1	2	3	4	5
	Amino nitrogen							
5	9.6 gm. as monohydrochloride (neutralized)	8.1	8.3	19.1	15.4	14.0	11.6	11.2
5	8.1 gm. as free base in 0.85% NaCl	11.3	11.3	17.4	16.0	14.8	13.6	12.9
5	7.9 gm. as free base in 0.85% NaCl	7.4	7.5	15.4	14.3	11.5	10.3	9.3
6 H	8.1 gm. as free base in 0.85% NaCl	7.3	7.3	17.8	13.7	11.9	10.4	10.3
6 H	8.1 gm. as free base in 0.85% NaCl	7.9	7.4	19.8	15.4	12.8	10.7	10.0
	Average for histidine	8.4	8.4	17.9	15.0	13.0	11.3	10.7
	Average for glycine (Doty and Eaton, '39)	9.8	9.5	29.4	13.9	11.2	11.6	10.8
	Average for lysine (Doty and Eaton, '39)	9.7	9.5	19.7	13.5	12.0	12.0	12.5
	Average for arginine (Eaton and Doty, '39)	10.2	10.2	17.0	12.6	10.4	9.9	10.0
	Urea nitrogen							
5	9.6 gm. as monohydrochloride (neutralized)	8.9	8.1	10.5	13.2	15.0	15.5	15.3
5	8.1 gm. as free base in 0.85% NaCl	9.0	9.0	10.2	12.6	15.3	16.7	15.2
5	7.9 gm. as free base in 0.85% NaCl	9.5	9.9	11.0	13.8	15.9	15.4	14.8
6 H	8.1 gm. as free base in 0.85% NaCl	7.2	7.7	9.3	10.1	11.6	10.2	8.9
6 H	8.1 gm. as free base in 0.85% NaCl	10.5	10.6	12.8	14.7	14.6	13.8	12.3
	Average for histidine	9.0	9.1	10.8	12.9	14.5	14.3	13.3
	Average for glycine (Doty and Eaton, '39)	11.6	12.0	15.7	19.3	19.9	17.6	15.9
	Average for lysine (Doty and Eaton, '39)	12.9	13.6	13.1	13.8	13.1	12.2	11.5
	Average for arginine (Eaton and Doty, '39)	11.0	11.1	15.4	18.5	20.9	20.1	18.5

the rapidity of removal of glycine from the blood stream represents not only an excretion comparable to that found with arginine, but also an extremely rapid rate of deamination.

The explanations given above are further confirmed by the values for blood urea nitrogen also shown in table 1. The

average changes in the blood urea nitrogen levels due to the administration of histidine roughly parallel those due to arginine. The value with arginine, however, does rise a little more rapidly than with histidine and remains somewhat higher at the end of the period. It should be stated that dog 5 was used for both histidine and arginine experiments, which would seem to make comparisons more nearly valid. The blood urea nitrogen values for glycine almost exactly parallel those for arginine for the first 3 hours but fall off more rapidly during the final 2 hours of the experiment. The figures obtained after lysine administration show no rise outside the limits of accuracy of the method and show a considerable decrease below control levels during the last 3 hours.

In only one (dog 7 H) of the six experiments shown in table 2 did the excretion of histidine equal that found with the other amino acids. Since, also, in this experiment alone, the extra urinary urea nitrogen appeared to exceed the extra total non-histidine urinary nitrogen, one must not take this amino acid figure too seriously. It is entirely possible that some forms of nitrogen, other than histidine nitrogen, might have been carried down along with the silver precipitate. Apparently histidine is more readily reabsorbed by the kidney tubules than is lysine or arginine. This conclusion is in accord with the finding that when these three compounds are fed under comparable conditions, histidine is the most rapidly absorbed from the gastro-intestinal tract (Doty and Eaton, '37).

The administration of histidine (with the one exception noted above) resulted in the excretion of a considerable amount of nitrogen in the urine which is neither urea nitrogen nor histidine nitrogen. A few determinations showed that no appreciable quantity of imidazole compounds other than histidine was excreted. The ammonia excretion was small and could account at most for only a small fraction of this undetermined nitrogen. In marked contrast to histidine, arginine gave very little undetermined nitrogen.

Because the blood urea nitrogen values had not returned to control level at the end of the experimental period, it is very

TABLE 2

Heat production and urinary nitrogen after intravenous administration of histidine

DOG NO.	WEIGHT	CONTROL PERIOD		EXPERIMENTAL PERIOD		URINE UREA N	EXCESS URINE UREA N	EXCESS TOTAL N (LESS N OF AMINO ACID EXCRETED)	AMINO ACID EXCRETED	AMINO ACID METABOLIZED	CAL./GM. EXTRA N	SPECIFIC DYNAMIC ACTION
		Average B.M.R.	Basal urine urea N	Amino acid used	Average metabolic rate							
	kg.	*Cal./hr.*	*mg./hr.*	*gm.*	*Cal./hr.*	*mg./hr.*	*mg.*	*mg.*	*gm.*	*m/Mol.*		*Cal./m Mol. amino acid metabolized*
5	10.5	16.55	55.8	9.6 gm. as histidine monohydrochloride neutralized with NaOH	17.79	132.1	406	665	0.78 as free base	9.66	9.34	0.642
5	10.7	——	66.4	8.1 gm. free base in 0.85% NaCl	——	140.4	411	726	0.66 as free base	9.79	——	——
5	11.1	——	70.5	7.9 gm. free base in 0.85% NaCl	——	168.4	612	967	0.78 as free base	14.57	——	——
6 H	7.3	16.77	57.4	8.1 gm. free base in 0.85% NaCl	17.76	169.2	604	1067	1.45 as free base	14.38	4.67	0.347
7 H	7.0	13.71	107.3	8.1 gm. free base in 0.85% NaCl	15.17	219.9	605	524	3.25 as free base	14.39	13.97	0.508
6 H	6.4	13.91	55.5	8.1 gm. free base in 0.85% NaCl	15.10	152.5	542	844	1.75 as free base	12.90	7.04	0.460
Average							530	799	1.44		8.75	0.489
Average for glycine (Doty and Eaton, '39)							714	961	3.02	51.0	12.91	0.244
Average for lysine (Doty and Eaton, '39)							458	—	6.65	16.4	——	——
Average for arginine (Eaton and Doty, '39)							182	185	2.92	3.25	22.35	0.399

difficult to estimate the amount of urea actually formed from the histidine administered. A rough approximation of the urea formed from the administered histidine but remaining in the tissues at the end of the experimental period may be made by applying the calculations used by Eaton, Cordill and Gouaux ('37). They established the basal rate of urea excretion previous to total nephrectomy and followed the increase of urea in the blood during a 5-hour experimental period. Calculations, making use of the weight of the dog, the rate of accumulation of urea nitrogen in the blood and the basal urea nitrogen production, indicated that the blood urea nitrogen was in equilibrium with approximately 70% of the body tissues of the dog. If one applies the same calculations to the histidine experiments, here reported, one finds the average urea nitrogen retention to be 240 mg. per dog experiment. This added to the average of 530 mg. per dog experiment excreted in the urine would give a total of 770 mg. of urea nitrogen formed from histidine. The amount of alpha amino nitrogen in the average quantity of histidine administered and retained was 593 mg. per dog. From this calculation it would appear that approximately 80% of the urea nitrogen formed could have come from simple deamination of the histidine molecule. Since the amount of undetermined (non-imidazole) nitrogen is large, it is further apparent that the imidazole ring was broken to some extent during the 5-hour period. This is more evident when one adds the calculated retained urea nitrogen in each experiment to the total non-histidine nitrogen excretion. The average per dog experiment of 1038 mg. (maximum 1278 mg.) nitrogen which could be accounted for in this fashion is well in excess of the average 593 mg. of alpha amino nitrogen administered but not excreted as such in the urine but comes far from accounting for the average of 1779 mg. of total histidine nitrogen administered and retained for metabolism. Evidently only a little more than half of the retained histidine nitrogen was transformed into urea or excreted as undetermined N within the 5-hour experimental period.

It is of interest to apply the same calculation to our results on arginine already published ('39) in order to have a more extensive basis of comparison of the metabolism of these two basic amino acids. The average total amount of arginine nitrogen administered, after deduction of that excreted in the urine, was 1852 mg. Half of this or 926 mg. should be transformed very rapidly into urea nitrogen by the action of arginase. The average excretion of urea for the 5-hour period amounted to only 182 mg. and the average amount calculated as formed but retained in the tissues would be 482 mg. per dog experiment. The total of 664 mg. as a rough approximation would thus fall far short of the quantity of urea to be expected by the primary action of arginase. These results would seem to have a significant bearing upon a consideration of the Krebs and Henseleit theory as regards the participation of the arginine-ornithine-citrulline cycle in normal urea formation. Although a final interpretation of the results with arginine must await a similar study with ornithine, we are probably justified in assuming that there is no significant accumulation of ornithine in the blood. Each molecule of ornithine contains two amino groups whereas each molecule of arginine contains only one such group available for the formation of nitrogen gas in the Van Slyke procedure. Similarly, the transformation of arginine to citrulline would involve no reduction in the amino nitrogen content of the system. It is conceivable, then, that the participation in the Kreb's cycle of a substantial fraction of the administered arginine would result in a slower rate of fall of the blood amino nitrogen as compared with the fall observed after administration of other amino acids. Our experiments furnish no evidence of such an effect. It is obvious, of course, that our results with arginine may require reinterpretation in the light of the suggestions that arginine may be metabolized after becoming an integral part of a protein molecule (Dirr, '39; Schmidt, Allen and Tarver, '40).

The over all change in the production of urea from the amino nitrogen of glycine or from histidine amino and non-amino nitrogen appears to take place more rapidly than does the

hydrolytic transformation of the guanido group of arginine into urea. It would seem improbable, therefore, that under the conditions of our experiment the latter mechanism is quantitatively important in the production of urea from glycine or histidine.

We must most emphatically state that our experiments do not disprove the theory of Krebs and Henseleit. They do, however, indicate that the urea formed from arginine, under the conditions used by us, could be formed entirely by mechanisms which do not involve either citrulline formation or a new synthesis of arginine. Regardless of any theoretical considerations, we have shown that more histidine is metabolized than arginine during a 5-hour experimental period when the two amino acids are administered in nearly equimolecular quantities.

Histidine exhibits a specific dynamic action about 50% greater than arginine but only about half that of an equal weight of glycine. On the basis of millimols deaminized the specific dynamic action of histidine is about twice that of glycine.

SUMMARY

Upon intravenous injection of l(—)histidine into the dog there is a prompt and long sustained rise in the urea nitrogen of the blood. In contrast to the results with glycine, lysine and arginine, there is but little excretion of unchanged histidine into the urine. Histidine is metabolized slightly faster than arginine as shown by the greater excretion of urea during the 5-hour experimental period.

Histidine exhibits a specific dynamic action which is about 50% higher than that shown by arginine, both on the basis of amount administered and amount metabolized. The total increase in heat production is about half as great as that produced by an equal weight of glycine.

No toxic effects of histidine were noted.

LITERATURE CITED

BLOCK, R. J. 1934 The determination of the basic amino acids in small quantities of protein by the silver precipitation method. J. Biol. Chem., vol. 106, p. 457.

DIRR, K. 1939 Aminosaürenabbau und Serumeiweisskörper; l(+)Arginin; ein Beitrag zum Krebsproblem. Zeitschr. f. physiol. Chem., Bd. 260, S. 65.

DOTY, J. R., AND A. G. EATON 1939 The heat production and blood and urine constituents after administration of d-lysine monohydrochloride to the dog. J. Nutrition, vol. 17, p. 497.

———— 1937 Metabolism of the basic amino acids. I. Rates of absorption in rats of the monohydrochlorides of d-lysine and l-histidine. J. Biol. Chem., vol. 122, p. 139.

EATON, A. G., AND J. R. DOTY 1939 The heat production and blood and urine constituents after administration of d-arginine monohydrochloride to the dog. J. Nutrition, vol. 17, p. 505.

EATON, A. G., S. C. CORDILL AND J. L. GOUAUX 1937 The metabolism of nephrectomized dogs. Amer. J. Med. Sciences, vol. 194, p. 214.

KREBS, H. A., AND K. HENSELEIT 1932 Untersuchungen über die Harnstoffbildg. im Tierkörper. Zeitschr. f. physiol. Chem., Bd. 210, S. 33.

RAPPORT, D., AND H. H. BEARD 1928 The effect of protein split products upon metabolism. III. Further investigations of the fractionated protein hydrolysates and of amino acids, and their relation to the specific dynamic action of the proteins. J. Biol. Chem., vol. 80, p. 413.

REINWEIN, H. 1928 Studien über den Mechanismus der spez.-dynam. Eiweisswirkung. Deut. Arch. klin. Med., Bd. 160, S. 278.

SCHMIDT, C. L. A., F. W. ALLEN AND H. TARVER 1940 Theory of protein metabolism: transformation of proteins. Science, vol. 91, p. 18.

VAN SLYKE, D. D. 1929 Manometric determination of primary amino nitrogen and its application to blood analysis. J. Biol. Chem., vol. 83, p. 425.

ON THE MANNER OF ACQUISITION OF FLUORINE BY MATURE TEETH[1]

MABEL W. PERRY AND W. D. ARMSTRONG
The Laboratories of Dental Research and Physiological Chemistry, The University of Minnesota, Minneapolis

(Received for publication August 22, 1940)

The demonstration (Armstrong, '37; Armstrong and Brekhus, '38 b) of significantly more fluorine in the enamel of sound teeth than in the sound enamel of carious teeth, furnishing the first substantial evidence that an optimum intake of fluorine is related to resistance to dental caries, has received support from two sources. First, field studies have shown a lower incidence of caries in areas of endemic mottled enamel (Arnim, Aberle and Pitney, '37; Klein and Palmer, '38; Dean, '38; Day, '40) and in regions in which the fluorine content of the drinking water is high irrespective of whether or not the teeth are mottled (Dean, Jay, Arnold, McClure and Elvove, '39). Second, the teeth of rats have been found to develop fewer caries when fluorine is added to the diet either during the time of development of the teeth (Cox, Matuschak, Dixon, Dodds and Walker, '39) or after the teeth are mature (Miller, '39; Hodge and Finn, '39).

The mechanism by which fluorine inhibits caries appears to be one of the following, operating alone or in conjunction with the other: (a) an optimum amount of fluorine in enamel confers properties upon it which make it more resistant to caries, (b) the presence of fluoride in the fluids of the oral cavity alters bacterial metabolism and inhibits enzyme action. The first is supported by the analyses of enamel of caries resistant

[1] This work was supported by a grant from the Carnegie Corporation and was aided by the personnel of the Works Progress Administration, Official Project No. 665-71-3-69, Sub-Project No. 344.

teeth (Armstrong, '37; Armstrong and Brekhus, '38 b); by the observation (Volker, '39) that fluorine incorporated in dental tissues reduces their acid solubility; by the continuing protection against caries afforded to the teeth of rats when they were fed extra fluorine only during the time of tooth development (Cox, Matuschak, Dixon, Dodds and Walker, '39); and by the interpretation placed upon the caries inhibiting action of fluoride occurring in rats deprived of salivary glands (Cheyne, '40). The second possibility was offered by Miller ('39), and Dean et al. ('39) tacitly assumed that this is the principal mechanism, but (a) cannot be excluded by the observations of the latter group of workers. The findings of Bibby and Van Kesteren ('39) with regard to the lessened acid production by oral bacteria in the presence of fluorine or fluorosed enamel and dentin may be interpreted in the light of either (a) or (b).

It has been proposed directly (Cox, Matuschak, Dixon, Dodds and Walker, '39) and by implication (Dean, Jay, Arnold, McClure and Elvove, '39) that fluorine be added to communal water supplies having a low content of this element as a means of reducing dental caries in the human. In this connection it would be of value to determine if fluorine can be incorporated into enamel other than at the time of the formation of the tooth. The possible avenues by which fluorine might enter the enamel of erupted teeth are: (a) by diffusion from the blood via the dentin and (b) by direct adsorption and incorporation into the mineral phase from fluids in contact with the tooth surface. Should (b) be feasible the fluorine content of enamel could be increased, when required, by topical application of soluble fluorides to the teeth. This method previously suggested by others (Volker, Hodge, Wilson and Van Voorhis, '40) would greatly reduce the dangers attendant upon the ingestion of excess fluorine and would eliminate the possibility of producing mottled enamel. While the present study was in progress it was shown (Volker, Hodge, Wilson and Van Voorhis, '40) in work also directed at the determination of the feasibility of (b), and in which the radioactive

isotope was used as an indicator, that fluorine is adsorbed from solution by powdered enamel in accordance with the requirements of the Freundlich adsorption isotherm. Since the powdered enamel used in this work possessed a greatly increased surface in comparison to that of a tooth in situ and possibly had altered properties the application of the results to in vivo conditions is uncertain.

The results to be reported will demonstrate that the fluorine content of enamel of molar teeth of mature living rats can be appreciably increased by fluorine which enters it by way of the surface of the teeth.

EXPERIMENTAL

Male albino rats which had been raised to maturity (average weight 250 gm.) on the same stock diet were fed for 60 days on commercial dog biscuits [2] and water, both supplied ad libitum. The food consumption was determined and the water intake measured at regular intervals. The water supplied over the 60-day period to the animals of the experimental group contained 20 p.p.m. of fluorine added as sodium fluoride and that consumed by the control animals was found, by analysis, to contain only a trace of this element. The animals of the experimental group were given, for 5 days before they were killed, distilled water in order to cleanse the crevices and sulci of their teeth of any fluorine which might have been occluded therein. After sacrifice of the animals the femurs and jaws were dissected out and boiled in distilled water until the soft tissues could easily be removed. The teeth, after removal from the jaws, were cleaned with the aid of dental instruments until inspection under magnification showed them to be free of soft tissue, bone, and debris. All specimens were stored in alcohol.

Inspection of the incisors of the experimental group revealed striations of alternating orange and lighter orange or white bands only discernible under magnification and more pronounced on the lower teeth. This effect of excess fluorine ingestion on the growing incisors of rats has been previously

[2] Purina Dog Chow.

described by several observers (McClure, '39) and is noted here only as evidence that the experimental animals received and absorbed a considerably increased amount of fluorine. The incisor teeth of the control animals were entirely normal in appearance.

TABLE 1

Fluorine intake of rats and fluorine contents of enamel, dentin and bone

ANIMAL NO.[1]	FLUORINE INTAKE FROM WATER	FLUORINE CONTENT				
		Incisor teeth		Molar teeth		Dry fat-free femur
		Dentin	Enamel	Dentin	Pooled enamel[2]	
	mg.	%	%	%	%	%
1C		0.0164	0.0190	0.0240		0.0248
2C		0.0153	0.0194	0.0250		0.0199
3C		0.0152	0.0181	0.0240		0.0288
4C		0.0135	0.0140	0.0236	(0.0169)	0.0189
5C		0.0161	0.0186	0.0235	(0.0176)	0.0224
6C		0.0149	0.0157	0.0240	(0.0177)	0.0198
7C		0.0171	0.0186	0.0233		0.0224
8C		0.0147	0.0194	0.0233		0.0186
9C		0.0154	0.0184	0.0243		0.0185
	Average	0.0154	0.0179	0.0238	0.0174	0.0215
1E	18.4	0.0340	0.0367	0.0244		0.0484
2E	24.5	0.0270	0.0230	0.0232		0.0476
3E	30.6	0.0300	0.0302	0.0236		0.0375
4E	37.3	0.0343	0.0341	0.0240	(0.0244)	0.0575
5E	36.4	0.0241	0.0261	0.0259	(0.0233)	0.0410
6E	33.6	0.0361	0.0366	0.0245	(0.0236)	0.0574
7E	36.4	0.0394	0.0329	0.0254		0.0508
8E	38.0	0.0384	0.0338	0.0247		0.0428
9E	28.5	0.0251	0.0256	0.0251		0.0353
10E	26.3	0.0250	0.0285	0.0240		0.0484
	Average	0.0313	0.0309	0.0246	0.0237	0.0466

[1] The letters C and E indicate animals of control and experimental groups.
[2] See text.

The molar teeth of each animal were pooled in separate lots and the incisor teeth were similarly combined in individual groups. Each lot was separately prepared for analysis (Armstrong and Brekhus, '37) and the enamel and dentin separated (Manley and Hodge, '39) by methods previously described. The bones were broken into bits, wrapped in cloth packets, extracted for 24 hours with alcohol in a Soxhlet apparatus,

dried, and pulverized. The analyses for fluorine were made by the method of Armstrong ('36) with certain modifications, the most important of which were the accurate measurement of the volume of indicator (sodium alizarine sulphonate) and the use of a fluorescent daylight lamp during the titrations.

The amount of enamel obtained from the pooled molar teeth of a single animal being so small (8 to 10 mg.), it was considered that analyses of the required accuracy of the molar enamel of individual animals would not be possible. Accordingly, equal weights of molar enamel from each animal were pooled and mixed, that from the experimental animals going into one sample and that from the controls into a second. The average of the analyses, performed in triplicate, of the pooled molar enamel of each group was the same as would have been obtained had the material from the individual animals been analyzed.

The fluorine intake of the rats during the experimental period and the results of analysis of their femurs, enamel, and dentin are shown in table 1. Sixteen samples of dentin and six of bone were analyzed twice. The average difference of the duplicate analyses was 2.8% and the maximum difference was 11.9%. Ten analyses for fluorine were made on a sample of pooled human enamel at various times during the course of this study. The average difference of the repeated analyses from the mean (0.0114% fluorine) was 2.6%.

DISCUSSION

The molar teeth of the control animals like the permanent teeth of the human (Armstrong and Brekhus, '38 a), with which they are comparable in development, were found to contain more fluorine in the dentin than in the enamel. The higher fluorine (Armstrong and Brekhus, '38 a) and magnesium (Armstrong and Brekhus, '37) contents of dentin of human teeth had been explained by the assumption that dentin is able, in contrast to enamel, to acquire these elements from the blood after the tooth is erupted. Further support for this hypothesis was obtained (Hevesy and Armstrong, '40) when it was shown

that radioactive phosphorus, subcutaneously administered to adult cats, could be demonstrated in appreciable quantities in the dentin but only in traces in the enamel. Since in the present study the molar dentin of the animals ingesting excess fluorine was not found to contain significantly more of this element than the molar dentin of the control animals, it is obvious that some revision is required of the hypothesis relating to the mechanism of interchange between dentin of mature teeth and the blood. With respect to fluorine it is evident, if such a mechanism exists, that it does not continue to act throughout the entire life of the tooth. Possibly the amount of fluorine found in molar dentin (of the order of 0.024%) represents its saturation content with respect to this element. Thus a secondary enrichment could occur until this amount is reached after which time no more fluorine can be deposited in the dentin of teeth in situ. That a secondary fluorine enrichment of teeth like rat molars which remain anatomically unaltered after eruption does occur at some stage subsequent to calcification is rendered probable by a consideration of the results of analysis of the enamel and dentin of the incisor teeth of the control animals. The incisor teeth grow continuously and therefore such teeth are newly formed and have had only a relatively short time to acquire extra fluorine. The fluorine contents of their enamel and dentin would be expected to be very nearly those present at the time of formation of the teeth and to represent, in general, the fluorine contents of recently deposited enamel and dentin. It will be noted that the fluorine contents of the enamel and dentin of the incisors of the control animals are about in the same proportion as the amount of mineral material in enamel and dentin and that the relationship of fluorine contents of the two fractions of these teeth is opposite to that of the molar teeth of the same animals. In consequence of the above considerations these results are evidence that enamel and dentin are not formed with a higher content of fluorine in the latter. Although there is no evidence to support the assumption, it is possible that conditions with respect to developing molar teeth of rats are different from

those pertaining to incisor teeth. If such were the case molar dentin might, at the time of formation, acquire more fluorine than enamel or incisor dentin.

Indirect evidence (Armstrong and Brekhus, '37), supported by direct experiment using radioactive phosphorus as an indicator (Hevesy and Armstrong, '40), has been interpreted to indicate that the composition of enamel of erupted mature teeth is incapable of alteration. However, the mean fluorine content of the molar enamel of the animals which received water containing 20 p.p.m. of fluorine was found to be significantly higher (36%) than that of the control animals. This difference is not due to contamination of the enamel fraction with dentin resulting from imperfect separation since the molar dentin of both groups of animals contained amounts of fluorine which were not significantly different. The latter fact is also evidence that the extra fluorine present in the molar enamel of the experimental animals did not enter it via the dentin. That it could have reached the enamel through the dentin without the fluorine content of the latter being increased would seem most unlikely. It is thus highly probable that the fluorine gained by the molar enamel of the test animals was absorbed through the surface of the enamel exposed to the oral cavity.

While the possibility cannot be excluded it seems certain that the fluorine which entered the molar enamel was derived from the drinking water as it was in contact with the teeth, a process which could continue until dilution with saliva greatly reduced the fluorine content of the oral fluids, rather than from fluorine secreted in the saliva. The relatively enormous amount of bone is known, from the work of numerous investigators, to absorb fluorine effectively from the blood and to retain it—a further example of which is seen from the results of analysis of the bones reported in table 1. Phillips, Hart and Bohstedt ('34) found it difficult to influence the fluorine content of another secretion, milk, by feeding proportionally larger amounts of fluorine than those received by the animals used in this study. In any event the possible increase of fluorine in the

saliva would not alter the conclusion that the extra fluorine entered the molar enamel from fluids in contact with the teeth.

It would appear that the very considerably increased fluorine contents of both fractions of the experimental incisor teeth were due, not only to the increased amount of fluorine received by the animals during the formation of the teeth, but also to adsorption and combination of this element through the surface of the enamel and dentin. The structure of dentin, which in the rat incisor is exposed on the posterior surface of the tooth, would likely facilitate the penetration of dissolved materials into it. On this basis one can account for the fact that the fluorine content of the incisor dentin of the test animals was increased from less than that of the enamel to a value equal to the latter even though the amount of fluorine in the enamel was considerably elevated.

If the findings of this study, insofar as they pertain to the molar teeth, are applicable to the teeth of the human it would appear possible to increase the fluorine content of at least the surface layer of enamel of human teeth by topical application of soluble fluorides. There is every reason to conclude, until positive evidence to the contrary is obtained, that fluorine once combined in enamel structure would remain there in large part. The fact that a high fluorine content of the enamel of the test animals was maintained for 5 days during which the animals consumed distilled water is evidence that the union is not a loose one. Fluorine present in the mineral phase of calcified tissues substitutes for hydroxyl in the structure of hydroxyapatite (Gruner, McConnell and Armstrong, '37). That the combination of fluorine in the fluor-apatite structure as it exists in enamel is stable is shown by the fact that normal human enamel contains (Armstrong and Brekhus, '38 a) 111 p.p.m. of fluorine, an amount which is far in excess of the fluorine content of any potable water or of the amount of fluorine which could reasonably be expected to occur in saliva or blood. An extreme example of this sort is seen in the case of a woman whose teeth had become mottled as a result of residing, during childhood, in a region of endemic mottled

enamel. Even though she had used Minneapolis city water for 22 years before the teeth were extracted the fluorine content (250 to 360 p.p.m.) of the enamel was found (Armstrong and Brekhus, '38 a) to be much higher than normal. The fact that powdered bone has been found an effective agent for the removal of fluorine from water (Smith and Davey, '39) is further evidence that the mineral salt of calcified tissues can acquire and retain fluorine against a concentration gradient.

SUMMARY

The molar teeth of rats fed an ordinary diet contain more fluorine in the dentin than in the enamel but the reverse obtains with respect to the incisor teeth.

The fluorine content of the dentin of molar teeth of mature rats is not increased when the animals are given water containing 20 p.p.m. of fluorine.

The above facts show that a secondary enrichment of dentin with fluorine occurs after its calcification and that the process does not continue indefinitely.

The fluorine content of the enamel of mature erupted teeth (rat molars) is increased by fluorine which enters it through the surface when rats are given drinking water containing 20 p.p.m. of this element.

LITERATURE CITED

ARNIM, S. S., S. D. ABERLE AND E. H. PITNEY 1937 A study of dental changes in a group of Pueblo Indian children. J. Am. Dent. Assn., vol. 24, p. 478.

ARMSTRONG, W. D. 1936 Microdetermination of fluorine. Elimination of effect of chloride. Anal. Ed., Ind. Eng. Chem., vol. 8, p. 384.

——— 1937 Fluorine content of enamel and dentin of sound and carious teeth. Proc. Am. Soc. Biol. Chem., J. Biol. Chem., vol. 119, p. v.

ARMSTRONG, W. D., AND P. J. BREKHUS 1937 Chemical composition of enamel and dentin. I. Principal components. J. Biol. Chem., vol. 120, p. 677.

——— 1938 a Chemical composition of enamel and dentin. II. Fluorine content. J. Dent. Research, vol. 17, p. 27.

——— 1938 b Possible relationship between the fluorine content of enamel and resistance to dental caries. J. Dent. Research, vol. 17, p. 393.

BIBBY, B. G., AND M. VAN KESTEREN 1939 Abstract of paper presented at Columbus meeting, Am. Assn. Adv. Sci.

CHEYNE, V. D. 1940 Inhibition of experimental dental caries by fluorine in the absence of saliva. Proc. Soc. Exp. Biol. and Med., vol. 43, p. 58.

COX, G. J., M. C. MATUSCHAK, S. F. DIXON, M. L. DODDS AND W. E. WALKER 1939 Experimental dental caries. IV. Fluorine and its relation to dental caries. J. Dent. Research, vol. 18, p. 481.

DAY, C. D. M. 1940 Chronic endemic fluorosis in Northern India. British Dent. J., vol. 68, p. 409.

DEAN, H. T. 1938 Endemic fluorosis and its relation to dental caries. Pub. Health Rep., U. S. P. H. S., vol. 53, p. 1443.

DEAN, H. T., P. JAY, F. A. ARNOLD, F. J. MCCLURE AND E. ELVOVE 1939 Domestic water and dental caries, including certain epidemiological aspects of L. Acidophilus. Pub. Health Rep., U. S. P. H. S., vol. 54, p. 862.

GRUNER, J. W., D. MCCONNELL AND W. D. ARMSTRONG 1937 The relationship between crystal structure and chemical composition of enamel and dentin. J. Biol. Chem., vol. 121, p. 771.

HEVESY, G., AND W. D. ARMSTRONG 1940 Exchange of radio phosphate by dental enamel. Proc. Am. Soc. Biol. Chem., J. Biol. Chem., vol. 133, p. xliv.

HODGE, H. C., AND S. B. FINN 1939 Reduction in experimental rat caries by fluorine. Proc. Soc. Exp. Biol. and Med., vol. 42, p. 318.

KLEIN, HENRY, AND C. E. PALMER 1938 Dental Caries in American Indian Children. Pub. Health Bulletin No. 239, U. S. Government Printing Office.

MANLEY, R. S., AND H. C. HODGE 1939 Dentistry and refractive index studies of dental hard tissues. I. Methods for separation and determination of purity. J. Dent. Research, vol. 18, p. 133.

MCCLURE, F. J. 1939 Fluorides in Food and Drinking Water. National Institute of Health Bulletin No. 172, U. S. Government Printing Office.

MILLER, B. F. 1939 Inhibition of experimental dental caries in the rat by fluoride and iodoacetic acid. Proc. Soc. Exp. Biol. and Med., vol. 39, p. 389.

PHILLIPS, P. H., E. B. HART AND G. BOHSTEDT 1934 The influence of fluorine ingestion upon the nutritional qualities of milk. J. Biol. Chem., vol. 105, p. 123.

SMITH, H. V., AND W. B. DAVEY 1939 The Development and Use of the Bone Filter for Removing Fluorine From Drinking Water. Technical Bulletin No. 81, Agricultural Experiment Station, University of Azirona, Tucson, Arizona.

VOLKER, J. F. 1939 Effect of fluorine on solubility of enamel and dentin. Proc. Soc. Exp. Biol. and Med., vol. 42, p. 725.

VOLKER, J. F., H. C. HODGE, H. J. WILSON AND S. N. VAN VOORHIS 1940 The adsorption of fluorides by enamel, dentin, bone, and hydroxyapatite as shown by the radioactive isotope. J. Biol. Chem., vol. 134, p. 543.

NUTRITIONAL REQUIREMENTS DURING THE LATTER HALF OF LIFE [1]

CLIVE M. McCAY, L. A. MAYNARD, GLADYS SPERLING AND HARLOW S. OSGOOD [2]

Animal Nutrition Laboratory, Cornell University, Ithaca, N. Y.

ONE FIGURE

(Received for publication August 12, 1940)

Few attempts have been made to determine the optimum diet for the adult after he has attained middle age. Such research is beset with difficulties foreign to similar studies for the first half of life. After the attainment of middle age the body becomes more and more subject to the diseases resulting from the regime followed during the first half. Furthermore, the diseases that accompany ageing appear. These diseases induce strains on the physiological mechanisms and make the evaluation of such factors as dietary variables very difficult. Nevertheless, the importance of the diet during the latter half of life must be of nearly the same order as during the first half. Therefore, it must be studied in spite of the handicaps imposed by the ageing process and in relation to these very changes and diseases which accompany ageing.

In the present studies the white rat has been employed because its life span is short and its diet during early life is well defined. The greatest limitation of the white rat and one which is little appreciated by most workers is the susceptibility, early in life, to diseases of the lungs. This is merely stated here but will be discussed elsewhere in our reports concerning the pathological aspects of our study.

[1] These studies were made possible through the Rockefeller Grant for Research in Longevity.

[2] Present address: Chemistry Department, Westminster College, New Wilmington, Pennsylvania.

EXPERIMENTAL

The effect of 8 and 30% protein levels upon longevity

We sought to determine whether the amount of protein modified the length of life when this was introduced as a variable in the middle of the life span. For this purpose, rats were kept until middle age upon our regular stock diet (Maynard, '30). When they had attained a mean age of 485 days, twenty-four were divided into two groups for blood studies. One was fed a diet having a percentage composition of casein 4, salt mixture 4, lactalbumin 3, butterfat 5, cellulose 2, sucrose 10, yeast 5 and cooked starch 67 while the other was fed the same diet with 42% starch and 29% casein.

Blood samples were taken from the hearts of these rats at the start and after they were on the diets for 105 and 187 days. At 105 days there was no significant difference in the value for the non-protein nitrogen level [3] and the means fell between 26 and 32 mg./100 cc. At the 187-day period, however, the N.P.N. had risen to 48 mg. for the rats fed the higher per cent of protein and remained at the previous level for those upon the 8% diet. This experiment was then repeated with rats of similar age. This time the protein level was not reflected in the N.P.N. at the end of 55 days, but a significantly higher level was found for the higher protein group at the end of 112 days.

A second study was run at the same time with the same diet to determine the effect of the protein level upon the life span. The mean length of life for the twenty-two males upon the lower protein diet was 679 $\pm$ 17 days and 641 $\pm$ 17 days for a similar number upon the higher level. These differences were not significant by statistical analysis.

The effect of 8, 14 and 20% protein levels

A third study was now undertaken using diets similar in composition to those of the first except that carotene was fed as an additional supplement to all groups. Diets containing

[3] The method was that of P. B. Hawk and O. Bergeim: Practical Physiological Chemistry, 9th ed., p. 368.

8, 14 and 20% of protein were used, shifting the casein level as previously. The mean age of the animals at the start was 445 days.

The results in terms of life span are summarized in table 1.

These data indicated no relation between the level of the protein and the length of life when tested statistically, with the exception of females fed the 8% level. These animals showed increased length of life.

Non-protein nitrogen was determined in the blood of eight rats from each group after they had been fed the diet for 116 and 238 days. At both periods the mean values were 30 to 34 mg./100 cc. for those fed the 8 and 14% protein levels but

TABLE 1

Mean life span and level of dietary protein in relation to the weight of ventricle and kidney

PROTEIN LEVEL	NUMBER OF ANIMALS		AGE IN DAYS AT DEATH		WEIGHT OF ONE KIDNEY		WEIGHT OF VENTRICLE	
	Males	Females	Males	Females	Male	Female	Male	Female
%					*gm.*	*gm.*	*gm.*	*gm.*
8	16	30	667	841	1.09(11)[1]	0.93(28)	1.25(11)	1.23(22)
14	15	30	640	769	1.32(14)	1.12(22)	1.57(11)	1.38(21)
20	18	35	654	770	1.32(12)	1.06(26)	1.63(11)	1.43(23)

[1] Values in parentheses show the number of organs weighed.

40 and 41 mg. for those allowed the 20% level. Many determinations of the daily excretion of albumin were made. Upon the highest protein level the values varied from 3 to 273 mg. However, the mean value for the 20% protein level was 82 $\pm$ 15 mg.; that for the two lower levels was 23 $\pm$ 6. Earlier attempts to correlate this excretion of albumin by the rat with the protein level of the diet had failed (McCay and Nelson, '26).

When animals died and were found while the tissues were fresh, weights of organs were obtained. The means of these values are assembled in table 1 for the ventricles and kidney weights. The weights of these organs in both sexes showed increases correlated with increased protein intake.

Fourth study using 8 and 20% protein

In the fourth study more attention was given to the albumin of the urine. This experiment was similar to the preceding ones and can be summarized in table 2.

Fifth study of protein level and such modifying factors as quality of the protein, exercise of the animals and restriction of body fatness

For this study two groups of male rats were kept until middle age upon the stock ration. At the time of starting the diets one group was 200 to 300 days of age and the other 400 to 450. After discarding all animals that did not appear in good health, 329 rats remained. These were divided into sixteen groups as evenly as possible from a consideration of body

TABLE 2

Level of protein and weight of organs

LEVEL OF PROTEIN	MEAN LIFE SPAN		MEAN KIDNEY WEIGHT		MEAN VENTRICLE WEIGHT		MEAN BODY WEIGHT	
	Male	Female	Male	Female	Male	Female	Male	Female
%	*days*		*gm.*		*gm.*		*gm.*	
8	673(22)	839(21)	1.20	1.02	1.25	1.23	265	194
20	716(22)	818(20)	1.75	1.14	1.54	1.44	272	192

weight, age and litter of origin. Thus each group contained either twenty or twenty-one individuals.

In order to get a better idea of the development of pathological changes before death, four from each of the sixteen groups were killed at intervals in the course of the study. The remainder of sixteen or seventeen rats in each group were kept until death, as in earlier studies.

The regimes for each of the sixteen groups are shown in table 4. These rats were housed in an air-conditioned room with the temperature maintained as nearly as possible at 78° F. and the humidity at 40 to 50%. All natural light was excluded from the room.

Throughout this study the short term "casein" is used to designate those groups receiving a mixture of lactalbumin and

casein as the major source of protein. The terms "thin" and "fat" are used to describe groups restricted and non-restricted, respectively, in their daily allowance of calories.

The purposes of these studies were (1) to compare the effects of high and low protein diets when consumed during the latter half of life, (2) to compare casein and liver as protein sources at two levels, (3) to determine the effect of forced exercise versus normal activity and (4) to compare animals allowed to

Fig. 1 Barrels equipped with a sewing machine belt running in bicycle rims and attached to a small motor and reducing gears, for exercising rats.

fatten with those kept thin in body weight during the latter half of life. The divisions described above made it possible to compare groups of twenty rats or to combine groups into large ones with 164 individuals for comparison. Thus all rats on high protein diets could be compared with all on low protein ones or all rats exercised could be compared with all not exercised.

Exercise was given by forcing the rats to run in rotating barrels shown in figure 1. The rate of rotation of the barrels

was first set very slow. Gradually the rate of rotation of the barrels was increased until the length of exercise period was extended to 2 hours daily. Exercise was started in February, 1937 and continued until November, unless rats became obviously sick. From our experience there is no doubt that middle-aged rats are killed by exercise if they are badly diseased or if they are not given a preliminary period of training.

In groups subjected to body weight restriction, the "thin" ones, the reduction of body weight was made by limiting the food eaten in these groups in order to hold the body about 10% below the maximum weight attained by individuals when

TABLE 3

Percentage composition of diets used in fifth study

	HIGH PROTEIN		LOW PROTEIN	
	Casein	Liver	Casein	Liver
Cooked starch	34	34	65	65
Casein	29	—	4	—
Lactalbumin	4	—	3	—
Sucrose	10	10	10	10
Cellulose	2	2	2	2
Butter	5	5	5	5
Lard	7	—	2	—
Yeast (irradiated)	5	5	5	5
Salt mixture	4	3	4	3
Dry liver	—	41	—	10

eating all the food desired and not exercising. The non-restricted groups were allowed all the food desired.

The four diets used in this study were constituted as shown in table 3. A supplement to this diet given to all rats was 1 mg. of carotene added to each kilogram of feed. In addition, every rat was given four yeast tablets per week. Each tablet weighed a third of a gram and carried 0.02 mg. of carotene.

Restricted and non-restricted groups all received the same amount of basal diet, but the non-restricted or "fat" rats were allowed all they wished of a mixture of sucrose 4, starch 4 and lard 1, in addition to the regular diet.

The salt mixture was prepared by mixing 3280 gm. of bone meal with an equal weight of limestone. To this was then

added 1640 each of KCl and NaCl. To this, in turn, was added 130 gm. of $MgSO_4 \cdot 7\ H_2O$ and 30 gm. of ferric citrate. The trace elements, consisting of $ZnSO_4 \cdot 7\ H_2O$, 5 gm., $CuSO_4 \cdot 5\ H_2O$, 1.6 gm., $MnSO_4 \cdot 5\ H_2O$, 10 gm., $AlCl_3 \cdot 6\ H_2O$, 5 gm., KI, 0.06 gm., were dissolved and sprinkled over the bone meal.

The rats of all sixteen groups were kept upon the diet until they died, with the exception of four from each group killed for study. Post mortem examinations were made in all possible cases. Reports of these examinations will be made elsewhere. Throughout this study x-ray photographs were used extensively in following calcification changes in cartilages, bone and tissues. These results will be summarized in a later article. All organs were weighed at the time of death, before they were fixed for histological study.

Statistical analyses were made of the data from each of the sixteen groups, as well as of combinations of these groups. All data are summarized in table 4. Conclusions are based upon these data.

RESULTS

Life span

None of the variables discussed in this report modify the length of life in a manner comparable to the retardation of growth discussed previously (McCay et al., '39). Of the sixteen groups, fourteen differ more than enough from others to give significant odds. Four combinations likewise differ to the same extent from four other combinations. In the groups given the higher protein, those fed liver outlived those fed milk proteins. Likewise those restricted in food intake in order to avoid excessive fatness of the body lived longer than those allowed to fatten at will. Restriction was more favorable in those fed liver at a high protein level than in those fed milk proteins.

The group that stands out from the others with respect to length of life is that fed a low protein diet with liver as the source of protein, kept thin and given exercise (13). This combination of factors was undoubtedly the most favorable.

TABLE 4

Diet, life span and organ weights

GROUP NO.	DIET	MEAN LIFE SPAN	P.E.M.[1]	MEAN BODY WEIGHT	P.E.M.	MEAN HEART WEIGHT	P.E.M.	MEAN KIDNEY WEIGHT	P.E.M.	MEAN LIVER WEIGHT	P.E.M.	MEAN SPLEEN WEIGHT	P.E.M.	MEAN TESTES WEIGHT	P.E.M.
		days	*days*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	High protein–casein–exercise–thin	614	23	272	7	1.57	0.08	1.62	0.08	11.8	0.9	0.97	0.06	1.36	0.18
2	High protein–casein–exercise–fat	563	16	320	13	1.31	0.05	1.62	0.15	12.3	0.6	0.75	0.05	1.71	0.13
3	High protein–casein–no exercise–thin	544	19	249	7	1.31	0.07	1.30	0.04	10.5	0.7	0.80	0.08	1.39	0.17
4	High protein–casein–no exercise–fat	572	21	311	14	1.31	0.07	1.53	0.08	11.5	0.7	0.87	0.09	1.13	0.09
5	High protein–liver–exercise–thin	663	24	260	8	1.40	0.07	1.57	0.06	11.4	0.6	0.81	0.09	1.24	0.12
6	High protein–liver–exercise–fat	574	17	244	7	1.55	0.09	1.60	0.07	12.8	0.7	0.75	0.07	1.15	0.13
7	High protein–liver–no exercise–thin	677	28	224	5	1.28	0.05	1.42	0.06	10.1	0.7	0.53	0.03	1.00	0.10
8	High protein–liver–no exercise–fat	609	22	240	7	1.32	0.07	1.37	0.07	11.4	0.5	0.53	0.03	1.30	0.14
9	Low protein–casein–exercise–thin	597	31	263	8	1.38	0.07	1.17	0.03	9.2	0.7	0.74	0.07	0.97	0.11
10	Low protein–casein–exercise–fat	590	22	269	10	1.32	0.05	1.23	0.05	10.7	0.7	0.62	0.05	1.26	0.09
11	Low protein–casein–no exercise–thin	641	28	229	8	1.46	0.09	1.19	0.06	8.7	0.5	0.62	0.04	0.95	0.07
12	Low protein–casein–no exercise–fat	581	19	313	11	1.32	0.07	1.29	0.03	11.5	0.9	0.68	0.08	1.86	0.15
13	Low protein–liver–exercise–thin	707	30	239	9	1.25	0.05	1.16	0.03	10.6	0.4	0.69	0.06	1.11	0.12
14	Low protein–liver–exercise–fat	621	28	253	8	1.19	0.05	1.07	0.03	10.5	0.8	0.85	0.11	1.31	0.12
15	Low protein–liver–no exercise–thin	587	28	257	8	1.25	0.07	1.13	0.13	10.6	0.5	0.49	0.05	1.25	0.13
16	Low protein–liver–no exercise–fat	597	22	243	7	1.29	0.05	1.17	0.09	10.6	0.6	0.65	0.05	1.23	0.11

[1] P.E.M. (probable error of the mean).

Among eight groups, this one was favored by odds ranging from 30:1 to 450:1.

Among the high protein groups, that fed liver and restricted without exercise outlived a similar group given exercise (see 7). In the case of animals exercised on the high liver diet the better group was that restricted.

In table 5 are shown the number alive in each group at intervals of 100 days. The 700-day period is rather a crucial one

TABLE 5

Number of rats alive at age intervals of 100 days and the ages attained by the oldest in each group

GROUP NO.	DIET	NUMBER ALIVE AT 100-DAY INTERVALS						AGE OF THREE OLDEST AT DEATH (DAYS)		
		500	600	700	800	900	1000			
1	High protein–casein–exercise–thin	14	9	4	1	0	0	769	792	889
2	High protein–casein–exercise–fat	11	8	2	0	0	0	681	708	743
3	High protein–casein–no exercise–thin	10	4	3	0	0	0	747	754	781
4	High protein–casein–no exercise–fat	13	6	3	1	0	0	730	738	862
5	High protein–liver–exercise–thin	13	10	7	4	0	0	851	853	871
6	High protein–liver–exercise–fat	12	7	2	0	0	0	652	717	751
7	High protein–liver–no exercise–thin	14	12	8	1	1	1	766	768	1131
8	High protein–liver–no exercise–fat	13	7	4	1	1	0	707	761	977
9	Low protein–casein–exercise–thin	13	9	5	2	1	1	783	832	1009
10	Low protein–casein–exercise–fat	13	9	4	1	0	0	712	732	856
11	Low protein–casein–no exercise–thin	12	11	8	3	1	0	804	839	900
12	Low protein–casein–no exercise–fat	12	9	3	0	0	0	712	772	787
13	Low protein–liver–exercise–thin	14	10	7	6	3	1	947	985	1062
14	Low protein–liver–exercise–fat	11	11	7	1	0	0	791	795	822
15	Low protein–liver–no exercise–thin	9	8	5	2	0	0	743	814	877
16	Low protein–liver–no exercise–fat	14	11	3	1	0	0	730	732	805

in the life of the rat since it corresponds to about 70 years in the life of man. At this time there were forty-seven rats alive in the restricted groups contrasted to twenty-eight in those not restricted. This is more marked for this variable than the 33:42 ratio found for high versus low protein.

There is little evidence to indicate that exercise plays an important role in the latter half of life. Perhaps the rat, being subject to lung disease, is not a good animal for such a study.

Exercise is probably advantageous for the healthy and disastrous for the sick rat.

As a rule the restriction of the calories of the diet is favorable for increasing the span of life, although in two groups this was not true. In general, rats kept underweight outlive those allowed to fatten as they will.

These data, as well as several earlier studies, afford little evidence that the level of protein in the diet affects the life span, if the limits are not too extreme. Of all groups, however, the best was that obliged to exercise, maintained thin, and fed a low protein diet supplied by liver. In most cases liver was more satisfactory as a source of protein than the milk proteins. Possibly this is subject to other interpretation, since the liver may have carried essentials such as unrecognized substances not found in the milk proteins.

As rats live to greater ages the body weight at the time of death becomes progressively less. This is only indirectly related to the diet, inasmuch as the diet modifies the life span. Among the sixteen groups studied, the mean difference between the maximum body weight attained and the weight at death varied from 129 to 194 gm. At the time of death the mean body weights for the different groups varied from 229 to 311 gm. No further conclusions can be drawn from the final weights as they were the result of the terminal disease for the most part.

The maximum body weight and life span

A spot diagram was prepared showing the relation of the maximum body weight attained at any time during life and the life span. The rats that lived the longest were those with intermediate body weights. This favorable zone for maximum life span lay between 350 and 450 gm. No animal whose body weight at any time in life exceeded 450 gm. was able to exceed 810 days for its life span. This was also true for animals whose body weights never exceeded 360 gm. For our colony the favorable zone lies within these limits of 350 to 450 gm. This is shown in the abridged table 6 prepared from the spot diagram.

Extreme age

Table 5 shows the age attained by each of the three oldest in each group. These data give some clue to the factors responsible. In the present study three individuals attained ages exceeding 1000 days. This is unusual among male rats of our colony. Two of these individuals were in the groups (9 and 13) fed low protein with restriction in food consumption and forced to exercise. The oldest three survivors were in such a group (13) with the additional variable of liver as the source of protein. The group also had the highest mean life span. It is, therefore, probable that the conditions under which this group lived were the most favorable, namely, low protein with liver

TABLE 6

Relation between the maximum body weight attained and the life span of rats in per cent of the weight group

MAXIMUM BODY WEIGHT	AGE AT DEATH (DAYS)					
	250–409	410–569	570–729	730–889	890–1049	1049 up
gm.						
Below 330	67	33	0	0	0	0
330 – 389	10	38	30	20	2	0
390 – 449	4	27	44	21	3	1
450 – 509	2	26	54	18	0	0
510 up	15	15	62	8	0	0

furnishing this protein. They also had exercise and were not allowed to become as fat as possible since their food intake was restricted.

For attaining extreme age, liver seems more favorable than the milk proteins. Exercise seems more favorable on a low protein diet than it does on a high one. Prevention of overfattening of the body seems important at both protein levels.

The weight of the heart

Only a few significant differences are found in the heart weights. The hearts of those fed the high liver diets were significantly heavier than those fed the low liver diet. This difference is also found between the small hearts of those fed a low

level of liver and the large hearts found when milk proteins at a high level with exercise and restriction were the other variables.

The weight of the kidneys

The well-known effect of protein upon the size of the kidneys is found throughout these data. Inasmuch as this is well known (Cowdry, '39) the interesting features to report here are that this enlargement resulted from the level of protein during the latter half of life and that these kidneys reflected the protein level at the death of the animal. The degree of wasting which accompanied the last diseases of old age did not obliterate the enlargement of the kidneys produced by the high protein diet. With one exception all low protein groups were very significantly different from corresponding high ones.

Of the groups fed high protein diets it was found that those which were forced to exercise (groups 1, 2, 5, 6) had heavier kidneys than those not exercised (groups 3, 4, 7, 8), whether they were fat or thin. The increase due to exercise was greatest in the thin animals fed casein—0.32 gm. (groups 1 and 3). Among individual groups in the higher protein series there were six that varied with odds greater than 30:1 while there was only one such case in the lower protein series. This suggests that several factors come into play when the kidneys are enlarged as a result of high protein diet. Among these, exercise, body fatness, and source of protein all play a part. Of course, none of these are as marked as the contrast between the small kidneys from the low protein diet and the larger ones from the high.

The weight of the liver

In considering organ weights no attempt has been made to relate the organ weight to the body weight at death inasmuch as groups were adjusted at the beginning of the experiment, in middle age for the rat, so that groups were equal in regard to size and weight of individuals. Relations of body weight and organ weight at the end represent the relative wasting of

the body in old age and the effect of the diseases involved at the end of life.

The livers of the animals fed the higher protein diet were heavier than those fed the lower level. This difference amounted to about 10% in contrast to that of the body weights which differed by about 3%. The restriction in the diet left its reflection in the weight of the liver even when rats died from natural causes. The livers of rats restricted in food intake were lighter in weight as one would expect. This is especially marked in the groups fed low protein diets with milk proteins as the source. The occurrence of small livers in the group fed milk proteins at a low level, without exercise and restricted is probably related to the low body weights at the time of death.

The weight of the spleen

The presence of so many significant variations in the weight of the spleen as a result of these different regimes is surprising. The total of these amounted to twenty-six, probably more than the number found in kidneys. These differences were much more prevalent in the high protein groups than in the low. The spleens of rats fed casein were larger than those fed liver at the higher level. The higher milk protein level produced larger spleens than the lower one. In the case of the higher protein level those groups exercised had larger spleens. Those fed higher levels of milk proteins had larger ones than those fed the higher amount of liver.

Krumbaar (Cowdry, '39) found that the weight of the spleen followed definite trends in relation to age but that there were great variations. This is what might be expected if factors of diet and regime can produce significant differences. The tendency of the spleen to reflect the state of nutrition must be much more marked than anyone has observed previously. Protein must be a very important dietary factor in modifying the spleen since so many significant differences were found for the higher level. The only one within the lower protein level occurred in those fed liver. This difference lay between the exercise variables in the non-restricted groups.

Testes

The significant differences in the weight of the testes are found in groups with levels of protein intake different from those characterizing any other organ differences. In contrast to the spleen the significant variations for the testes are found for the most part in the groups fed low levels of protein. In the lower protein levels those fed restricted intakes had smaller testes with the effect much more marked in the milk protein groups. Those fed the higher levels of casein had larger testes than those fed the lower. Within the groups fed the higher casein level, exercise was the factor producing the larger testes. From the whole picture, protein seems to be the dominant factor in determining the size of the testes at death. At lower levels of protein, the restriction of calories becomes very important.

The lipids of the organs

In three of the four animals killed from each group, the total lipids, the lipid phosphorus and the cholesterol were determined upon the fresh tissues. Two series of significant differences were found. Animals restricted in food intake had lower levels of both cholesterol and total lipids in both the heart and liver, while the lipid phosphorus values were about the same. The livers of animals fed liver diets were richer in all three lipid fractions than those fed casein diets, while the hearts showed no differences. The high and low levels of liver in the diet produced about the same lipid composition in the organs of rats. None of these differences were found in the kidneys.

Comparison of weights of organs of sacrificed rats with those of rats dying of natural causes

To determine if the killing of a small number of healthy rats in the course of a life span experiment affords any clue to the weight of the organs of rats from a given group at the time of death, correlation coefficients were determined between the

weights of organs of those killed and those that died in old age in each group. The values for "r" were the following:

ORGAN	r	P.E.
Liver	0.66	±0.09
Heart	0.005	
Testes	−0.033	
Kidneys	0.63	±0.10
Spleen	0.31	±0.14

From the killing of a few representatives of given groups one can anticipate the weight of certain organs such as the kidneys or liver at the time of natural death. To a limited extent even the weight of the spleen can be foretold roughly. On the other hand, the testes and even the heart must be much affected by the terminal disease since their weights bear little relation at the time of death to the weights of comparable organs from members of the same group killed while in good health.

SUMMARY

Five studies have been completed to determine the effect on rats of different diets and regimes introduced in middle life. The protein level of the diet during the latter half of life does not affect the life span significantly when the protein constitutes 8 to 30% of the diet. At the higher level of protein intake, the non-protein nitrogen of the blood increases and the heart and kidneys become larger. The albumin of the urine varies widely but is roughly correlated with the protein level of the diet. The factors of most importance during the latter half of life, so far as the life span is concerned, are those which determine the degree of fatness of the body. Rats kept underweight by restriction of calories outlive those allowed to fatten. Exercise is of minor importance in comparison with body fatness. The optimum conditions for a long life proved to be thin bodies, exercise, and a low protein diet with this protein provided by liver.

A simple method for exercising animals is described.

The weights of the organs of rats killed while being subjected to different regimes in late life correlate with those of

the rats that die a natural death in the case of the liver and kidneys but not in the case of the heart and testes.

Inasmuch as the major disease that afflicts rats during the latter half of life is one that involves the lungs, it is questionable whether the effects of high protein intake on rats are comparable to those on man.

LITERATURE CITED

COWDRY, E. V. 1939 Problems of Ageing. Williams and Wilkins, Baltimore, p. 586.

MAYNARD, L. A. 1930 A diet for stock rats. Science, vol. 71, p. 192.

McCAY, C. M., L. A. MAYNARD, G. SPERLING AND L. L. BARNES 1939 Retarded growth, life span, ultimate body size and age changes in the albino rat after feeding diets restricted in calories. J. Nutrition, vol. 18, p. 1.

McCAY, C. M., AND V. E. NELSON 1926 Metabolism and vitamin A. J. Metabolic Res., vol. 7–8, p. 199.

SEVERE CALCIUM DEFICIENCY IN GROWING RATS[1]

I. SYMPTOMS AND PATHOLOGY

MURIEL D. D. BOELTER[2] AND DAVID M. GREENBERG
Division of Biochemistry, University of California Medical School, Berkeley

THREE FIGURES

(Received for publication August 15, 1940)

Although the literature concerning the role of calcium in metabolic processes is extensive, no one has reported studies of what might be called uncomplicated calcium deficiency. Most of the dietary regimes previously employed either were not so low in calcium as can now be obtained, or were not carried along for a sufficient period of time to exhaust the animal's calcium supply. It is the purpose of the present paper to report studies in which these objections have been met. This study was undertaken as a sequel to the investigation of the effects of magnesium deficiency carried out by Tufts and Greenberg ('38).

EXPERIMENTAL METHODS

Diet. The composition of the basal diet and of the control and low calcium salt mixtures has been published in a previous paper (Kleiber, Boelter and Greenberg, '40).* The diets employed in this study were similar in composition to those used by Tufts and Greenberg ('38) in their work on magnesium deficiency. The salt mixtures were prepared so as to contain

[1] Aided by grants from The Rockefeller Foundation and The Christine Breon Fund for Medical Research. Technical assistance was furnished by the personnel of W.P.A., official project no. 65-1-08-62. A preliminary communication has been previously published (Greenberg, Boelter and Knopf, '39).

[2] The material of this paper was taken from a thesis submitted by Muriel D. D. Boelter to the Graduate Division in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May, 1940.

* Table 1 in the paper of Kleiber, Boelter and Greenberg ('40) giving the basal diet and salt mixtures used in this study, contains two errors. The values of NaCl in the Ca-deficient and control diets are transposed, and the dosage of the crystalline vitamin supplements is per kilo of diet.

a minimum number of constituents in order to facilitate their purification. All salts used were recrystallized to free them from contaminating substances as far as was feasible. The rice bran extract,[3] used as a supplement to supply certain members of the B group of vitamins, was re-extracted with 50% alcohol.

Analysis of the diets showed that the control contained 546 mg. Ca, 84 mg. Mg, and 436 mg. P, while the usual calcium-low food contained 9.4 mg. Ca, 85 mg. Mg, and 425 mg. P per 100 gm. food. Low calcium diets containing about 4 and 20 mg. Ca per 100 gm. were also employed in certain of the experiments. The diet with only 4 mg. of Ca per 100 gm. food was obtained by greater care in purifying the individual components.

The selection and care of the rats and the methods used for the analysis of blood, tissue, and body constituents were the same as those employed by Tufts and Greenberg ('38).

SYMPTOMS AND SEQUELAE

1. Growth

The first noticeable effect of the calcium deficient diet is upon the growth rate. In general, there is no other noticeable manifestation of the deprivation for about 2 months. When the animals are allowed to feed ad libitum, the difference between the weight gained by the deficient and the control animals becomes pronounced at the end of the second or third week. At this time the controls gain over 20 gm. per rat per week, whereas the corresponding gain of the deficient animals is 10 gm. or even less. After 4 or 5 weeks, the deficient animals usually cease to grow, and, in fact, may even decline in weight. These facts are illustrated in the growth curves given in figure 1.

The decreased rate of growth of the calcium-deficient rats is, to a large extent, the result of decreased appetite. The average food consumption of rats fed the deficient diet is

[3] Vitab B.

6 to 7 gm. per rat per day as against about 12 gm. for the controls. The weight of the calcium-depleted animals is also adversely affected by the fact that their metabolic rate is higher than that of the controls (Kleiber, Boelter and Greenberg, '40).

Within limits, the time required for the appearance of the symptoms of calcium deprivation, including the retardation

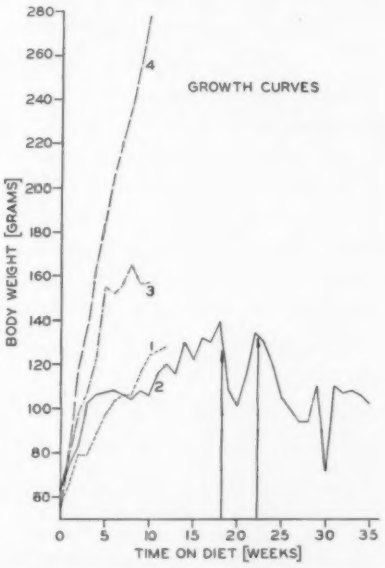

Fig. 1 Effect of the calcium level of the diet on the growth of rats. Average starting weights varied from 55 to 63 gm. Curves are drawn from data of groups of six or more animals. The arrows indicate the time of onset of paralysis in several animals of the group. Curve 1, about 4 mg. Ca per 100 gm. diet; curve 2, about 10 mg. Ca per 100 gm. diet; curve 3, about 20 mg. Ca per 100 gm. diet; curve 4, control, about 550 mg. Ca per 100 gm. diet.

of the growth rate, varies with the calcium content of the diet, taking longest with the diet containing 20 mg. Ca and least with the 4 mg. Ca per 100 gm. food (fig. 1).

Between the weights of about 40 to 100 gm., the size of the animals at the time they are placed on the synthetic low cal-

cium diet merely affects the ultimate weight which the rats attain. The similarity between the growth curves of rats placed on the deficient diet at ages of between 25 to 42 days, respectively, is shown in figure 2. The larger animals maintain

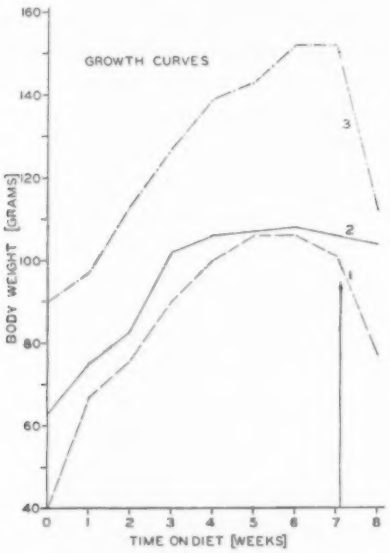

Fig. 2 Influence on growth of weight of animal at beginning of calcium deprivation. Calcium content of the deficient diet is about 10 mg. per 100 gm. diet. The curves are drawn from data of groups of six animals each. The arrow indicates the time of onset of paralysis of the hind quarters of some animals in each group. The average weight and starting age of the animals were: curve 1, 40 gm., 25 days; curve 2, 63 gm., 32 days; curve 3, 90 gm., 42 days.

their advantage in weight throughout the experimental period, but suffer the same consequences at approximately the same time as the younger group. This is indicated by the arrow in figure 2.

2. Symptoms of calcium deficiency

The calcium-deficient animals usually show no outstanding characteristics different from the normal for from 7 to 10

weeks, and some show no ill effects for the whole of their survival time.

In the first place, the animals show a decreased sensitivity and reactivity. They do not exhibit the usual curiosity shown by the control rats, but are inert and quiescent, moving around the cage very little, and evidencing no interest in the proceedings around them. *In no instance was tetany induced in the rat by lack of calcium in the diet alone.* Such potent stimuli as the galvanic current or the sound of an air blast failed to elicit the convulsive seizures of tetany.

After 6 to 9 weeks, there may be a loss of hair, varying from a slight thinning of the coat to a baldness extending over the whole of the dorsal surface of the animal. The possibility exists that the loss of hair may be the result of a disfunction of the vitamins of the B [4] group, since administration of a high level of riboflavin during the first few weeks on the deficient diet favors the maintenance of a healthy coat and may altogether prevent the appearance of baldness.

After about 2 months or more, any one or all of the more drastic symptoms may appear. These include prostration, paralysis of the hind extremities, black feces, and hemorrhages from the anus and from the toe-nail bed. Only a relatively small percentage of the animals succumb spontaneously to prostration and paralysis. The incidence is recorded in table 1.

The paralytic foot-drop of one or both hind legs is most probably the secondary effect of hemorrhages in the central nervous system. The paralysis varies in severity depending upon the degree of calcium depletion of the rat. In a severe case of paralysis, the hind legs are outstretched backward or to one side. If the animal is not entirely helpless, but can move a little, it drags the hind limbs, pushing itself forward with its fore limbs. Immediately after an animal becomes

[4] That the lack of calcium, and not an inadequacy of vitamins, is the ultimate cause of the observed deficient syndrome is proved by the fact that animals maintained on a restricted control ration, so that they actually consume significantly less food than the deficient rats, show none of the symptoms attributed to calcium lack, but are normal in every respect except rate of growth.

paralyzed, it is extremely sensitive to touch and squeals continuously even if handled gently.

Sometimes the rat may be completely prostrated for a week, and, being unable to consume any food, may consequently suffer a great loss of weight. This is illustrated by curve 2 in figure 1. The time of onset of paralysis is indicated by the arrow.

TABLE 1

Incidence of death and paralysis on low calcium diet of a total of seventy-eight rats

TIME ON DIET IN WEEKS	DEATHS			SPONTANEOUS PARALYSIS		
	Number[1]	% of total	Summation of %	Number	% of total	Summation of %
3	2	2.6	2.6			
4	1	1.3	3.9	1	1.3	1.3
8	5	6.4	10.3	5	6.4	7.7
9	1	1.3	11.6	2	2.6	10.3
10	3	3.9	15.5	1	1.3	11.6
11	11	14.0	29.5	2	2.6	14.2
12	4	5.1	34.6	1	1.3	15.5
13	6	7.7	42.3	1	1.3	16.8
14	2	2.6	44.9	1	1.3	18.1
15	0	0	44.9	2	2.6	20.7
17	4	5.1	50.0	1	1.3	22.0
18	1	1.3	51.3	1	1.3	23.3
19	2	2.6	53.9			
20–23	5	6.4	60.3			

[1] Animals that did not die were used for experimental purposes after being 12 to 20 weeks on the diet.

More than half of the animals suffering spontaneous paralysis will succumb within 24 hours. Of those surviving the initial shock, the majority recover in 2 to 3 weeks, after and during which time they are, of course, liable to repeated attacks. Except for the animals that die of inanition resulting from prolonged prostration, death appears to be due to shock.

Frequently, the skull of a dead rat in a cage will be gnawed through and the brain eaten by the surviving animals.[5] No

[5] Magnesium deficient rats show a preference for eating entrails of deceased cage mates, although often every part of a dead animal will be devoured but the skin, which, found in the morning in a neatly rolled up bundle, may be the only remaining evidence of the cannibalistic feast.

other part of the carcass has ever been attacked, and there is no evidence to indicate that a prostrated animal is ever attacked before its decease.

The neurological condition and the poorly calcified bones, which do not provide adequate skeletal support, affect the posture and movements of the animals in various ways. The rats show an unsteady gait, walking with a waddling movement or hopping in order to protect either one or both hind legs. They have difficulty in standing erect, and usually squat on their haunches with the hind legs tucked under. They may walk stiff-legged, putting down each foot with great care. Sometimes they push themselves backward or in a circle, using the fore limbs only and pivoting on the hind quarters. Conditions similar to the above are passed through on recovery from a case of severe paralysis, or they may result from mild spontaneous attacks not actually involving paralysis of the hind limbs.

The bones of the calcium deficient animals are easily broken, and spontaneous fractures of the leg bones are common.

3. Effect of electrical stimulation

The prostration and paralysis may be induced at a much earlier date (4 weeks) than they occur spontaneously by means of mild, short galvanic shocks from an induction coil, and there is 100% response to this stimulus after 6 to 7 weeks on the calcium-low diet. The electrical stimulus causes an immediate collapse and the subsequent paralysis of the calcium-deprived rat. The animal falls on its back or rolls over on its side, the head and hind limbs being drawn together with the latter flexed against the abdomen. Even if it is not completely prostrated, it shows little ability to right itself or to grasp objects with its paws. The animal is not unconscious as in tetany, but it responds poorly and sluggishly to stimuli. As in spontaneous cases, the animal is extremely sensitive to touch immediately after it has recovered from the initial shock, but within 24 hours the surface of the body loses sensibility and the animal no longer shows any response to even painful

superficial stimuli. The fore limbs of the rat are not involved in the reaction to the electric shock, but remain relatively normal throughout. Severely prostrated animals are incontinent. The appearance of an animal suffering from a severe case of paralysis is illustrated in figure 3.

The amount of current sent through the animal is extremely small and of brief duration. The deficient rats are affected practically instantaneously, whereas a normal animal is merely annoyed and irritated by even prolonged electrical stimulation. The stimulus of the sound of an air blast, which is so

Fig. 3 A calcium deficient rat photographed when it had been 9 weeks on the deprivation regime, 6 days after the induction of paralysis by galvanic stimulation. Note the helpless paralytic condition of the hind limbs, the encrusted nares, the size, and complete prostration of the rat. This animal died 2 days later, weighing 82 gm. Hemorrhagic areas were observed in the lungs, brain, liver, intestines, and gluteal muscle.

effective in causing tetanic convulsions in animals reared on a low magnesium diet, does not disturb the calcium-deprived rats at all.

Calcium-deficient rats that have survived a convulsive dose of strychnine also develop a paralysis of the hind quarters.

4. Longevity

The span of life is greatly reduced and the mortality rate is greatly increased in the animals on the low calcium diet. In a series of observations on seventy-eight animals, 60% died in from 3 to 23 weeks, while, in the same period, not a single death occurred among the animals on the control diet. The mortality statistics and the incidence of the onset of spontaneous seizures of prostration and paralysis are given in table 1.

5. Recovery

Animals that survive the shock of either a spontaneous or electrically induced paralytic condition may recover completely, probably due to resorption of the hemorrhagic clot, even though they continue to ingest the calcium-low food. Recovery is hastened, however, if the animals are transferred to the normal control diet. In some instances there is a permanent loss of the use of one of the limbs even though the animal recovers in every other respect. These animals drag the affected limb along in much the same manner as is sometimes observed in human cases surviving a hemoplegia.

Four weeks after the control level of calcium is added to the rations of the deficient rats, they are no longer susceptible to the induction of paralysis by the electric shock. This was determined on animals that had been on the low calcium regime for from 10 to 14 weeks, and had suffered either spontaneous or induced paralysis and hemorrhages before they were placed on control food. For the first few weeks on the recovery diet, they react as usual, the effect of the galvanic current decreasing as the time on the normal diet progresses. Immediately after the rats are transferred to the control diet, the amount of food consumed returns to the normal level and likewise the weight gain increases to 20 to 30 gm. per rat per week. The animals were found to have a normal blood calcium concentration after 7 weeks on the calcium-adequate diet. Eventually it is impossible to distinguish such rats from normal animals in appearance and reactions.

6. Effect of injection of calcium salts

On the other hand, injections of calcium salts do not protect the depleted animals from the consequences of the galvanic shock and coincident paralysis produced by it. In fact, invariably the calcium injection causes the death of the rat within 24 hours, even if the animal is not subjected to a galvanic shock. The outstanding autopsy finding to which the cause of death can be ascribed is the rupture of the ventricle of the heart in a longitudinal line from the base to the apex. In the calcium low animals, the ventricular wall is abnormally thin,

and the added calcium ion in the circulation possibly causes the heart to stop in systole, and engorge the ventricle with blood, so that it bursts. The lungs, auricles, and all of the blood vessels of, and those leading to, the heart are engorged. Most of the animals die within 3 to 5 hours after the injection.

Similar results were obtained with doses of 5 mg. of calcium in the form of the chloride, lactate, and gluconate, per 100 gm. of body weight. Eleven out of fourteen calcium-deficient animals, and not a single one of the controls, died following injection of the calcium salts. Twice this dosage of calcium had no effect on the corresponding control rats.

Hoff, Smith and Winkler ('39) have recently reported that calcium chloride caused the death by circulatory failure of normal animals within a few hours after its injection. The salt produced fibrillation of the heart before death, and autopsy revealed a dilated heart and engorged pericardium. These authors state that calcium has a direct depressant effect on the heart muscle.

7. Autopsy and histological findings

The conspicuous feature found on examination of the calcium-deprived animals after death from shock and paralysis is the hemorrhage which may occur in practically every internal organ of the body. Massive hemorrhagic areas are frequently noted in the muscles of the gluteal region. The liver and the auricles of the heart, or both, may be engorged with blood, and not infrequently the latter may rupture. Hemorrhages have been found in the lungs, brain, bladder, bone, gastrointestinal walls, nail matrix, and in the peritoneal cavity. The intestinal contents are usually very dark, and frequently the rats have diarrhea. The intestinal hemorrhage appears to originate in the duodenum, as it is at this point that the intestines become black.

The skull is practically uncalcified and paper thin. The rest of the skeleton also is very poorly calcified. It has already been pointed out by Kleiber, Boelter and Greenberg ('40) that calcium-deficient rats are shorter, and that they have a smaller skull and finer skeleton than the pair-fed controls.

Histological studies [6] revealed striking differences between the bones of normal and low calcium rats. The contrast was especially well shown in the cortical bone of the tibial diaphysis where, in the depleted animals, porosis was very pronounced. The compact lamellation present in the normal bone was absent in the bones from the calcium-deficient animals, except for very limited thin periosteal and endosteal shells. Between these narrow periosteal and endosteal zones of almost normal appearing bone there were trabeculae of apparently poorly calcified osseous tissue. The nuclei of the osteocytes found in this tissue are much larger and stain more lightly with hematoxylin than those of the typical osteocyte. These cells were embedded in a fibrous matrix arranged in disorderly fashion, suggesting that the normal Haversian systems had been completely disrupted. Between the trabeculae a very cellular soft tissue had replaced the bone matrix. These areas showed blood vessels engorged with blood, and small hemorrhagic zones appeared occasionally. Many osteoclasts were observed with their cytoplasmic processes continuous with the remaining bone-trabeculae, resembling somewhat the condition produced by certain stages of dosage with parathyroid hormone. Also present in the resorption areas were many cells indistinguishable from fibroblasts as well as many typical osteoblasts, and where communications had been established with the marrow cavity, myeloid tissue had penetrated between the trabeculae.

Exophthalmos has been observed in rats that have been on the diet for as long as 14 weeks. This is probably a symptom of their higher metabolic rate. In the later stages of the deficiency, the animals may suffer a ringed-eye dermatitis, and several rats were observed to have developed a cataract of one eye.

Histological examination of animals suffering paralysis shows hemorrhagic areas in the following regions of the central nervous system: cerebrum, medulla, brain stem, about the choroid plexus of the third ventricle, in the lower right temporal pole of the brain, and, only occasionally, in the spinal

[6] We are indebted to Dr. William R. Lyons for the description of the histologic picture of the bones.

cord. Evidence of hemorrhage is found in the lung, liver and kidney. There are small interstitial blood leaks in the voluntary muscle. The other organs examined were found to be normal.

In the brain no abnormality has been observed in the cerebellum. Special myelin staining shows no evidence of demyelinization of any part of the nervous system. However, the spinal cord itself is very soft and gelatinous in consistency on gross comparison with the normal. Practically no cellular reaction is found in the area of the intracerebral hemorrhages, whereas the medulla and choroid plexus of the third ventricle show hemosiderin phagocytosis.

Examination of the intestines reveals the presence of many brass-colored (hematoxylin-eosin stain) particulate bodies, located in the villous capillaries, in the intervillar spaces, and at the muco-submucosal junction of the large and small intestines. The particles are only occasionally phagocytized in these regions of the intestinal wall, but a diffuse deposition of the bodies is also observed in the fixed sub-mucosal macrophages. The particulate matter does not give the reaction for iron with the Prussian blue stain. There is much denudation of the intestinal wall of an occasional animal.

The black feces, encrusted nares, and traces about the urito-anal region of a bloody urine found in animals suffering from prostration and paralysis are, therefore, external evidence of hemorrhages that have occurred within the body.[7]

In explanation of the hemorrhagic state, it may be pointed out that calcium ion has long been associated with cell permeability, its effect on capillaries being to increase vascular tone and to decrease permeability by causing capillary constriction (Cantarow, '33). Eppright and Smith ('37) attributed the observed blood extravasation from the nares on a salt-free diet to a capillary fragility due to the deficiency of calcium. Histological examination indicates that possible alterations in the

[7] Because of the well-known fact that vitamin C deficiency causes hemorrhages in susceptible animals, experiments were carried out to determine the effect of the addition of ascorbic acid on the hemorrhagic condition suffered in calcium deficiency. No alleviation of the condition produced by a lack of calcium was found on feeding the experimental rats an abundant amount of ascorbic acid.

blood vessel walls are produced by our calcium-deficient regime.

8. Urine

A striking characteristic of the severe calcium deficiency is the output of a very large volume of urine. In one trial the deficient animals averaged an excretion of about 25 cc. of urine per day as against 8 cc. for the controls. The average specific gravity was 1.02 and 1.06, respectively. Microscopic examination showed the presence of some erythrocytes and very many

TABLE 2

Comparison of urinary volume and specific gravity in normal and calcium-deprived rats

		VOLUME	SPECIFIC GRAVITY
		cc.	
Calcium deficient	Rat 1	23.9 ± 1.9 [1]	1.02 ± 0.003
	Rat 2	24.5 ± 2.2	1.02 ± 0.002
Control	Rat 5	4.4 ± 0.6	1.08 ± 0.006
	Rat 6	10.5 ± 0.6	1.05 ± 0.006

[1] The figures represent the average of weekly values and their mean deviations of the mean for a period of 9 weeks on the experimental diet.

crystals in the urine of the calcium-deficient rats. A summary of the data on urinary volume and specific gravity is given in table 2. Data on the mineral composition of the urine will be published in connection with a study of the calcium balance in this condition. On the other hand, the output of feces by the calcium deficient rats is very scanty. The great reduction in the amount of calcium phosphate and calcium soaps in the intestinal tract is responsible for this.

CONCLUSIONS

The chief results of this investigation may be summed up in the following conclusions. A severe deficiency of calcium in the diet of the rat leads to muscular weakness and collapse rather than to tetany. The outstanding effect of the low calcium regimen is the susceptibility of the animal to hemor-

rhages. The bleeding may be the result of a disturbance of the role calcium plays in cell permeability, or may be due to changes in the vascular walls.

SUMMARY

1. Growing rats reared on a synthetic diet containing about 10 mg. calcium per 100 gm. food developed a condition characterized by the following syndrome: (a) retardation of growth; (b) decreased food consumption; (c) high basal metabolic rate; (d) reduced activity and sensitivity; (e) osteoporosis or low calcium rickets; (f) abnormal posture and gait; (g) susceptibility to internal hemorrhage which results in prostration, paralysis of the hind quarters (frequently fatal), encrusted nostrils, bleeding from the anus, and black, diarrheal feces; (h) a large increase in the volume of the urine; and (i) reduced span of life.

2. The hemorrhages and their consequences of prostration and paralysis are easily induced by a mild galvanic shock.

3. Rats suffering from the effects of calcium deprivation again become normal in appearance, reactions, and chemical composition a few weeks after they are supplied food with an adequate amount of calcium.

4. The injection of calcium salts almost invariably causes the death of the calcium-deprived animals within a few hours.

LITERATURE CITED

CANTAROW, A. 1933 Calcium metabolism and calcium therapy. Philadelphia.

EPPRIGHT, E. S., AND A. H. SMITH 1937 The influence of specific mineral deficiencies on the growth of body and organs of the rat. J. Nutrition, vol. 14, p. 21.

GREENBERG, D. M., M. D. D. BOELTER AND B. W. KNOPF 1939 New observations on the effects of calcium deprivation. Science, vol. 89, p. 18.

HOFF, H. E., P. K. SMITH AND A. W. WINKLER 1939 Electrocardiographic changes and concentration of calcium in serum following intravenous injection of calcium chloride. Am. J. Physiol., vol. 125, p. 162.

KLEIBER, M., M. D. D. BOELTER AND D. M. GREENBERG 1940 Fasting catabolism and food utilization of calcium deficient rats. J. Nutrition, vol. 19, p. 517.

TUFTS, E. V., AND D. M. GREENBERG 1938 The biochemistry of magnesium deficiency. I. Chemical changes resulting from magnesium deprivation. II. The minimum magnesium requirement for growth, gestation, and lactation, and the effect of the dietary calcium level thereon. J. Biol. Chem., vol. 122, pp. 693, 715.

SEVERE CALCIUM DEFICIENCY IN GROWING RATS[1]

II. CHANGES IN CHEMICAL COMPOSITION

MURIEL D. D. BOELTER[2] AND DAVID M. GREENBERG
Division of Biochemistry, University of California Medical School, Berkeley

FOUR FIGURES

(Received for publication August 15, 1940)

A description of the symptoms and pathological changes produced by diets very low in calcium in growing rats has been given in the first paper of this series (Boelter and Greenberg, '41). The accompanying chemical changes in blood, tissues and bone form the subject matter of the present report.

With the exceptions noted below, the methods used for the analysis of blood, tissue and body constituents were the same as those employed by Tufts and Greenberg ('38).

RESULTS

1. Blood

There is a marked progressive lowering of the calcium concentration of the blood during the course of the extreme calcium deprivation. This is brought out in the curves of figure 1. The majority of the data were obtained from animals on a deficient diet containing approximately 10 mg. Ca per 100 gm. food. However, the three values, constituting curve 2, are from rats that received the 4 mg. Ca diet. These figures are among the lowest obtained and were reached much earlier in

[1] Aided by grants from the Rockefeller Foundation and The Christine Breon Fund for Medical Research. Technical assistance was furnished by the personnel of W.P.A. (official project no. 65-1-08-62).

[2] The material of this paper was taken from a thesis submitted by Muriel D. D. Boelter to the Graduate Division in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May, 1940.

the course of the experiment. The ultimate result with either of the above calcium levels, however, is the same, so that ordinarily the less highly purified diet was employed.

The serum calcium starts to fall rapidly after the second week on the 10 mg. Ca diet, reaches its low level of around

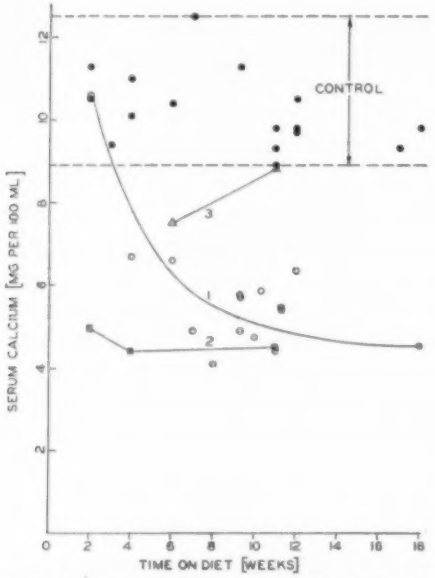

Fig. 1 The decrease in serum calcium concentration during calcium deprivation. Analysis made upon composite blood samples of two to four animals each. Results are expressed in milligrams of calcium per 100 cc. of serum. Curve 1 (open circles), about 10 mg. Ca per 100 gm. diet; curve 2 (open squares), about 4 mg. Ca per 100 gm. diet; curve 3 (open triangles), about 20 mg. Ca per 100 gm. diet; control range (solid circles), between the two horizontal dotted lines, about 550 mg. Ca per 100 gm. diet.

5 mg. per 100 cc. in about 8 weeks, and remains equally subnormal during the rest of the lifetime of the animal. The two points forming curve 3, in figure 1, represent analyses from rats receiving a ration which contained as much as 20 mg. Ca per 100 gm. food. They are decidedly higher than all the corresponding serum calcium values on the lower calcium-

containing diets. However, they are still below the lower limits of the control range. The time at which prostration and paralysis first can be produced by electrical stimulation corresponds with the attainment of a low serum calcium concentration.[3]

The low blood calcium levels are of interest because never has there been noted a case of tetany among the hundreds of animals reared by us that must have had a blood calcium of between 4 to 6 mg. per 100 cc., a concentration which is supposed to be the cause of "low calcium" tetany in parathyroidectomized animals.

Low values of the serum calcium concentration unaccompanied by any signs of tetanic convulsions have been reported in animals suffering from a variety of pathological conditions, including parathyroid deficiency (Godden and Duckworth, '35; Pal and Singh, '38; Salvesen and Linder, '23; Shohl and Bing, '28; Shohl and Wolbach, '36; Templin and Steenbock, '33; and Wade, '29). Many authors have come to the conclusion that factors other than calcium deficiency are involved in the production of tetany, and that a diminished level of serum calcium is not invariably associated with tetany.

The changes in inorganic serum phosphorus concentration are shown in figure 2. The inorganic phosphorus of the calcium-deficient animals falls at first more or less parallel to the calcium concentration, but it returns to the initial level after about 2 months on the calcium-low diet.

A summary of the blood levels of certain other constituents besides calcium and phosphorus is given in table 1. The serum and red corpuscle magnesium of the calcium-deprived rats remain within the normal range throughout the whole time of the experiment. The blood sugar and hemoglobin are the same for the deficient and normal rats. The serum phosphatase level, which might be expected to be altered because of

[3] Paralysis could be induced by electrical shock in parathyroidectomized rats maintained on the low calcium diet when their serum calcium concentration was normal.

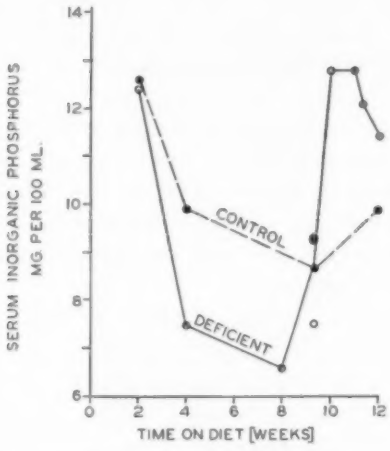

Fig. 2 Changes in serum phosphorus of animals reared on calcium deficient and normal diets. Data expressed as milligrams phosphorus per 100 cc. serum. Analysis upon pooled blood samples. Deficient (solid line and open circles), about 10 mg. Ca per 100 gm. diet; control (broken line and solid circles), about 550 mg. Ca per 100 gm. diet.

TABLE 1

Composition of the blood in calcium deficiency

CATEGORY OF INTEREST	DEFICIENT			CONTROL		
	No.	Range	Mean	No.	Range	Mean
Serum magnesium [1]	8	2.2–3.6	2.8	8	2.5–4.5	3.4
Red corpuscle magnesium [1]	8	4.3–9.9	7.6	8	6.3–8.8	7.8
Blood sugar [1]	2		84	2		85
Serum phosphatase [2]	3	108–123	115	5	98–132	112
Hemoglobin [3]	3	79– 80	80	4	79– 85	82

[1] Analytical values expressed in milligrams per 100 cc.

[2] Values expressed in tyrosine units (Greenberg, Lucia and Weitzman, '40). Several determinations by Bodansky's method ('33) yielded average values of 24.9 for deficient and 21.5 for control rats.

[3] Values expressed in per cent according to the Newcomer method ('23), (100% = 16.9 gm. hemoglobin).

the defects in the skeleton, was found also to be essentially the same as that of the calcium-supplied controls.

In these analyses blood sugar was determined by the method of Miller and Van Slyke ('36), phosphatase by the method of Greenberg, Lucia and Weitzman ('40) with disodium phenyl phosphate as substrate, and, in several instances, by Bodansky's method ('33) with sodium-β-glycero-phosphate as the

TABLE 2

Calcium, magnesium, and phosphorus content of certain tissues in extreme calcium deficiency. Data in milligrams per 100 gm. fresh tissue

	DEFICIENT				CONTROL			
	No.	Range	Mean	σ^1	No.	Range	Mean	σ^1
				Calcium				
Muscle	19	3.1–19.2	7.2	0.9	17	4.0–16.8	7.9	1.0
Kidney	24	1.0–10.9	5.6	0.5	24	3.7–10.0	6.2	0.3
Heart	20	3.0–28.0	10.9	1.3	19	2.7–19.7	8.1	0.9
				Magnesium				
Muscle	15	20.7–36.0	30.4	1.1	14	19.8–38.6	30.2	1.4
Kidney	18	18.9–27.1	23.6	0.5	18	15.1–37.9	23.8	1.2
Heart	14	19.8–36.9	25.8	1.2	13	17.6–25.9	22.3	0.75
				Phosphorus				
Muscle	14	86–142	110	3.5	4	81–130	108	10
Kidney	14	117–279	153	10	7	123–213	153	11
Heart	13	86–142	110	5	3	87–134	113	—
Liver	17	100–164	140	4	7	88–148	120	7
Brain	15	124–170	143	4	6	123–174	149	7

[1] σ = mean deviation of the mean = $\sqrt{\frac{\Sigma(\bar{X}-X)^2}{n(n-1)}}$

substrate. Hemoglobin levels were determined by the Newcomer method ('23).

The only striking alteration, therefore, that has been observed in any of the blood constituents, is the reduction of the serum calcium concentration.

2. Soft tissues

Table 2 contains a statistical summary of the calcium, magnesium, and phosphorus contents, respectively, of certain of the soft tissues. These represent analyses at various stages

of the deficiency covering the time period from about 14 to 84 days. No striking changes from the normal are noted in the amount of calcium in the body tissues at any time during the progress of the deprivation. The results indicate that there may be some trend toward depletion of the calcium content of the muscle and kidney, but the variability is such that the

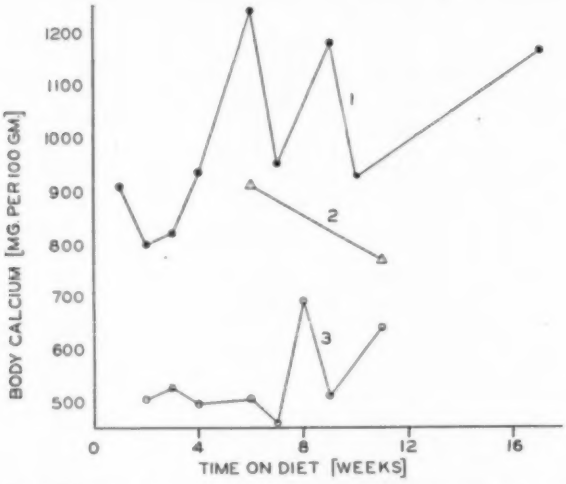

Fig. 3 Variation in calcium content of whole rat carcass with time during calcium depletion. Data are expressed in milligrams calcium per 100 gm. fresh carcass. The points represent weekly averages of analytical data. Curve 1 (solid circles), control, about 550 mg. Ca per 100 gm. diet; curve 2 (open triangles), about 20 mg. Ca per 100 gm. diet; curve 3 (open circles), about 10 mg. Ca per 100 gm. diet.

difference between the calcium deficient and control animals is not statistically significant. On the other hand, the calcium is tenaciously retained by the heart.

3. Mineral content of the whole body

The changes in body calcium are shown in the curves of figure 3. The points of the curves represent the average calcium percentage of groups of animals sacrificed at weekly intervals. After as little as 2 weeks on the calcium-low diet,

the body calcium is already reduced to about two-thirds the content of the control rats. Between 8 and 12 weeks on the diet, there is a noticeable increase in the per cent calcium of the deprived-rat carcass. The body calcium content of the control rats increases with growth, so that at 6 to 7 weeks the deficient level becomes actually less than half of the normal.

On the diet of 4 mg. Ca per 100 gm. food, the calcium depletion is even more drastic, the body calcium reaching the extremely low value of 313 mg. per 100 gm. of carcass in the rat reared for 11 weeks on this diet. This is considerably below the values found on the usual deficient diet at any time. On the 20 mg. Ca diet, depletion of the body calcium is very much slower, so that at 6 weeks on the diet, the body calcium is nearly within the control range.

The average values of the per cent magnesium in the bodies of the calcium-deficient animals are always below those of the normal rats of the same age. The lowered total body magnesium reflects the low content of magnesium in the bones of the calcium-depleted animals. The changes in body magnesium are shown in figure 4. During the initial stages of calcium depletion, there is an increase in whole body magnesium, but after 2 months on the calcium-low regime, the per cent of magnesium decreases, paralleling the drop observed in the control animals.

4. Composition of bone

A statistical summary of the chemical data obtained from the analysis of the bones of the deficient and control animals is recorded in table 3.

The bones of the calcium-deprived animals are poorly calcified, but the composition of the bone mineral is not essentially altered. The poor degree of mineralization of the bones of the deficients is indicated by the ash content being about 40% below that of the controls. Because of the low ash content, the percentage of calcium in the deficient bones is only about one-half that of the controls. The magnesium and phosphorus are about one-third lower than the normal. In percentage of bone

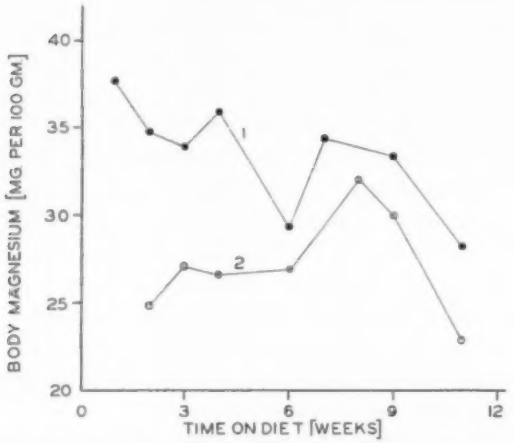

Fig. 4 Changes in total body magnesium during the course of calcium depletion. Data are expressed as milligrams magnesium per 100 gm. fresh carcass and are obtained from the weekly averages of the analytical values. Curve 1 (solid circles), control, about 550 mg. Ca per 100 gm. diet; curve 2 (open circles), about 10 mg. Ca per 100 gm. diet.

TABLE 3

Composition of bone

	ASH	CALCIUM		MAGNESIUM		PHOSPHORUS	
		Ash	Bone	Ash	Bone	Ash	Bone
	%	%	%	%	%	%	%
	Calcium deficient (21 analyses, 14–84 days on diet)						
Range	21.6–53.9	18.2–40.6	6.7–19.9	0.39–1.96	0.15–0.62	10.1–20.5	3.6–9.8
Mean	39.9	29.7	11.8	0.94	0.35	16.9	6.9
σ [1]	1.7	1.2	0.7	0.09	0.03	0.5	0.4
	Control (11 analyses, 7–84 days on diet)						
Range	54.0–69.2	32.3–43.7	18.6–28.3	0.56–0.90	0.37–0.59	13.4–20.3	9.0–13.1
Mean	65.0	38.1	24.7	0.75	0.48	16.3	10.5
σ [1]	1.3	1.3	0.9	0.04	0.03	0.6	0.4
	Recovered [2]						
Range	72.7–73.8	29.6–32.4	21.5–23.9	0.46–0.48	0.33–0.37	12.3–12.9	8.9–9.5
Mean	73.2	31.0	22.7	0.47	0.35	12.6	9.2

[1] σ = mean deviation of the mean = $\sqrt{\frac{\Sigma(\bar{X}-X)^2}{n(n-1)}}$

[2] Data for two rats that were reared on the control diet for about 22 weeks after having been deprived of calcium for 10 weeks following weaning.

ash, the mean calcium value is only 16% below normal, the magnesium about 25% higher than the control, and the phosphorus is unchanged.

Analysis of the bones of two animals given the control food for 22 weeks after they had been 10 weeks on the calcium-low diet following weaning revealed a normal percentage of ash and calcium, a significant reduction in phosphorus, and a very low content of magnesium.

SUMMARY

1. Severe calcium deprivation causes a rapid reduction in the serum calcium concentration, and a decrease in the calcium and magnesium content of the whole rat carcass. The serum inorganic phosphorus, magnesium, and phosphatase, red corpuscle magnesium, and the blood sugar do not deviate from normal. There is the usual content of hemoglobin in the blood. The soft tissues maintain their normal mineral content.

2. The bones of the deficient animals are greatly demineralized. The per cent of ash and calcium of the bone is only about one-half the normal. There is a lesser decrease in the phosphorus and magnesium percentage. In the bone ash, the contents of calcium, magnesium, and phosphorus are slightly low, high, and normal, respectively.

LITERATURE CITED

BODANSKY, A. 1933 Determination of serum phosphatase. Factors influencing the accuracy of the determination. J. Biol. Chem., vol. 101, p. 93.

BOELTER, M. D. D., AND D. M. GREENBERG 1941 Severe calcium deficiency in growing rats. I. Symptoms and pathology. J. Nutrition, vol. 21, p. 61.

GODDEN, W., AND J. DUCKWORTH 1935 Variations in serum-magnesium and in the partition of serum calcium in normal parturition and in milk fever. Biochem. J., vol. 29, p. 445.

GREENBERG, D. M., S. P. LUCIA AND H. G. WEITZMAN 1940 Estimation of serum phosphatase activity with disodium phenyl phosphate. J. Lab. and Clin. Med., vol. 25, p. 634.

MILLER, B. F., AND D. D. VAN SLYKE 1936 A direct micro-titration method for blood sugar. J. Biol. Chem., vol. 114, p. 583.

NEWCOMER, H. S. 1923 A new optical instrument for the determination of hemoglobin. J. Biol. Chem., vol. 55, p. 569.

PAL, R. K., AND N. SINGH 1938 Effect on rats of supplementing the South Indian diet with calcium and phosphorus. Indian J. Med. Research, vol. 25, p. 693.

SALVESEN, H. A., AND G. C. LINDER 1923 Observations on the inorganic bases and phosphates in relation to the protein of blood and other body fluids in Bright's disease and in heart failure. The relation between calcium and protein of serum in tetany due to parathyroidectomy. J. Biol. Chem., vol. 58, pp. 617, 635.

SHOHL, A. T., AND F. C. BING 1928 Rickets in rats. Rickets and tetany. Am. J. Physiol., vol. 86, p. 633.

SHOHL, A. T., AND S. B. WOLBACH 1936 The effect of low calcium-high phosphorus diets at various levels and ratios upon the production of rickets and tetany. J. Nutrition, vol. 11, p. 275.

TEMPLIN, V. M., AND H. STEENBOCK 1933 The effect of vitamin D on calcium concentration in adult rats maintained on low calcium diets. J. Biol. Chem., vol. 100, p. 209.

TUFTS, E. V., AND D. M. GREENBERG 1938 The biochemistry of magnesium deficiency. I. Chemical changes resulting from magnesium deprivation. II. The minimum magnesium requirement for growth, gestation, and lactation, and the effect of the dietary calcium level thereon. J. Biol. Chem., vol. 122, pp. 693, 715.

WADE, P. A. 1929 Clinical and experimental studies on calcium and cholesterol in relation to the thyroid-parathyroid apparatus. Am. J. Med. Sci., vol. 177, p. 790.

STUDIES ON RIBOFLAVIN AND THIAMIN IN THE RUMEN CONTENT OF CATTLE [1]

CHAS. H. HUNT, C. H. KICK,[2] E. WISE BURROUGHS, R. M. BETHKE, A. F. SCHALK AND PAUL GERLAUGH

Department of Animal Industry, Ohio Agricultural Experiment Station, Wooster

(Received for publication August 26, 1940)

Bechdel, Honeywell, Dutcher and Knutsen ('28) in connection with studies on the synthesis of the vitamin B complex in the rumen of the cow reported that the fermented rumen content contained more of the vitamin B complex than the feed of the animal. These investigators also found that one bacterium of the genus Flavobacterium was about 90% predominant in the rumen microflora and that the feeding of the dead bacteria to rats on a vitamin B complex free ration resulted in increased growth. From these findings the authors concluded that the vitamin B complex was produced in the rumen of the cow by bacterial action and that the results offered an explanation as to why Bechdel, Eckles and Palmer ('26) were able to grow cattle to maturity and have them produce normal offspring on a ration that carried an insufficient amount of the vitamin B complex to support growth and well-being in rats. Since this work was done, it has been shown that the vitamin B complex, as originally postulated, is made up of several factors, parts of which have been chemically identified.

The fact that Bechdel and associates ('28) found a chromogenic bacterium to predominate in the microflora of the rumen suggested to us that possibly riboflavin was being synthesized in the rumen by bacterial action and not vitamin B_1 (thiamin) alone.

[1] Published with the permission of the Director of the Ohio Agricultural Experiment Station.

[2] Deceased.

In connection with another study that is in progress at this institution, which makes use of an artificial rumen fistula in steers, the opportunity was afforded to study the possible synthesis of some of the fractions of the vitamin B complex in the rumen of the animal. The present report deals with riboflavin and thiamin of the feed and rumen content.

EXPERIMENTAL

Biological assays

The experimental procedure consisted of feeding weighed amounts of the feed the steers received or the dried content of the rumen to rats, as sources of riboflavin and thiamin. The rats were confined to wire cages when 35 to 45 gm. in weight and fed a vitamin B complex free ration, consisting in percentage composition of vitamin-free casein [3] 20; sucrose, 71; salt mixture, 4; hydrogenated cottonseed oil,[4] 3; and cod liver oil, 2.

For the riboflavin studies, the above ration was supplemented with daily additions of 10 μg. of thiamin and a water soluble fraction of yeast [5] from which the greater part of the riboflavin had been removed. The amount of yeast extract fed daily was equivalent to 1.5 gm. dried yeast. This amount of yeast extract plus thiamin resulted in an average gain of 10 gm. in forty-six rats during the assay period of 6 weeks. When adequate amounts of riboflavin (40 μg.) were fed in addition to the yeast extract and thiamin, average gains of about 20 gm. per week for 6 weeks resulted. The yeast extract in amounts of 0.25 gm. dried yeast daily also cured acrodermatitis in rats.

When the rats had attained a satisfactory weight (plateau in weight curve in 2 to 3 weeks) as evidenced by daily weighings, they were transferred (equal number of males and females) to individual cages and the finely ground samples of feed or dried rumen content were fed in separate dishes as the source of riboflavin. A negative control group was included

[3] Labco.

[4] Crisco.

[5] Northwestern Yeast Co., Chicago, Ill.

in each trial. At the same time different levels of synthetic riboflavin were fed in addition to the yeast extract and thiamin to obtain a calibration or reference curve (table 1) which was used to calculate the riboflavin content of the products under study (table 2). To assure that the growth response induced by feeding the dried ingesta in these studies was a measure of its riboflavin content and not of other growth factors, a group of rats was fed 600 mg. of dried (16 hours) ingesta daily from trial 5 in addition to the yeast extract, thiamin and 40 μg. riboflavin. The results presented in table 1 show no growth increase from the feeding of the dried ingesta.

TABLE 1

Weight increments in rats fed varying amounts of riboflavin

SUPPLEMENT	NUMBER OF ANIMALS	AMOUNT FED DAILY	AVERAGE INCREASE IN WEIGHT 6 WEEKS	AVERAGE INCREASE IN WEIGHT OVER NEGATIVE CONTROLS
		μg.	*gm.*	*gm.*
	8	2.5	32	25
Positive control	8	5.0	53	46
Synthetic ribo-	8	10.0	83	76
flavin (Merck)	8	20.0	111	104
	8	40.0	126	119
600 mg. ingesta	5	40.0		117
Negative controls	8	0.0	7	

In the thiamin studies the above vitamin B complex free ration was supplemented daily with 600 mg. of autoclaved yeast as the source of the vitamin B complex factors other than thiamin. In trial 5 the autoclaved yeast was replaced with 1.5 cc. of the yeast extract (1.5 gm. yeast) and 40 μg. of riboflavin. Rats which received either the autoclaved yeast or yeast extract plus riboflavin all died of polyneuritis in the course of 3 to 4 weeks. When the growth curves of the rats had exhibited a weight plateau (3 to 4 weeks) the animals were transferred (equal number of males and females) to individual cages and given weighed amounts of the finely ground feed or dried ingesta as the source of thiamin. The supplemental feed-

ing was continued for 6 weeks. Negative control groups were included in each series.

The steers used in trials 1, 2, 3, 4, 6, and 7 were mature animals and had been on their respective rations for several weeks before the samples of ingesta were collected. The daily ration in case of trials 1, 2, 3, and 4 consisted of 8.0 pounds of yellow corn, 8.0 pounds alfalfa hay, and 1.5 pounds of protein supplement. In trials 6 and 7 the steers were fed alfalfa hay exclusively. Two young steers weighing about 400 pounds were used in trial 5 and each was fed daily 2.5 pounds yellow corn, 3.5 pounds chopped alfalfa hay, and 0.5 pound protein supplement. One of these steers was fed its portion of corn whole and the other ground. Samples of ingesta were collected from each animal after they had been on the above ration for 2 weeks, then the rations were reversed and another sample of ingesta taken from each animal after 10 days of feeding. The ingesta from the two animals fed whole corn and the same animals fed ground corn were then combined for drying and feeding.

Method of collecting samples

In all trials except trial 5 the content of the rumen was sampled by removing 15 to 20 pounds of the ingesta from various parts of the rumen at different intervals after feeding. The sampling in all but three trials extended over 3 or more days. After sampling and compositing, the ingesta were dried in shallow pans before an electric fan in a dark room. In some cases part of the composited ingesta were incubated for 12 or 48 hours at 37°C. and then dried. The samples in trial 5 were obtained by removing the entire rumen content of the steer at each collection period. The ingesta were thoroughly mixed, sampled, and the remainder returned to the rumen. The fresh samples of ingesta were treated with 95% ethanol so that the final concentration was about 45% by weight and then stored in a cool, dark place until all samples had been collected and composited, after which they were dried by the same method as in the other trials.

The feed in all trials was sampled on the day before and during the days when collections of ingesta were made. The samples for each trial were then finely ground and composited in the same proportion as they were fed to the animal. All samples of feed and dried rumen content were stored in the dark.

RESULTS AND DISCUSSION

The results of the riboflavin studies are presented in table 2. In all instances (trials 1, 3, 4, and 5) where the steers had been fed a ration of yellow corn, alfalfa hay, and a protein supplement, the dried ingesta which had been in the rumen 12 or 16 hours showed a greater riboflavin content than the feed. In two out of the three trials (trials 2, 4, and 5), when the corn-hay-protein supplement ration was fed, the 4-hour dried ingesta sample showed an increased riboflavin content when compared with the feed. The incubation of the 4-hour ingesta sample in two trials showed an increase in riboflavin content when incubated for 48 hours (trial 2) but no increase when incubated for 12 hours as in trial 4. The 16-hour ingesta sample (trial 3) incubated for 48 hours did not show an increase in riboflavin due to incubation.

It is of interest that in both trials (6 and 7) where alfalfa hay served as the only feed of the steers no increase in the riboflavin content of the dried rumen content was observed either in the 4- or 16-hour sample. Furthermore, there was no increase in the riboflavin content of the 4-hour ingesta (trial 7) sample after incubating for 48 hours at 37°C. Kick and associates ('38) have reported that the rumen content of steers fed alfalfa hay exclusively is alkaline in reaction, whereas if grain and a protein supplement are fed with alfalfa hay the rumen content is acid in reaction. Whether the reaction of the rumen content or some other factor was concerned in the results obtained on the two different types of rations used in these studies is not clear and requires further investigation. The results, in general, show that a synthesis of riboflavin takes place in the rumen of the steer when a mixed ration of corn, alfalfa hay, and a protein supplement is fed. These observa-

TABLE 2

The comparative riboflavin content of the feed and the dried ingesta of the rumen

TRIAL	MATERIAL	TIME[1] IN RUMEN	TIME INGESTA INCUBATED AT 37°C.	DAYS INGESTA COLLECTED	RATS	AMOUNT FED DAILY	AVERAGE GAIN OVER CONTROLS	ESTIMATED RIBOFLAVIN CONTENT PER GRAM
		hours	*hours*	*no.*	*no.*	*mg.*	*gm.*	*μg.*
1	Feed				6	600	29	5
	Ingesta	12		1	6	600	42	7
2	Feed				8	300	20	7
	Ingesta	4		1	8	300	20	7
		4	48	1	8	300	34	11
3	Feed				8	300	15	5
	Ingesta	16		3	8	300	27	8
		16	48	3	8	300	25	8
		16		9	8	300	25	8
4[2]	Feed				8	300	15	5–6
					8	600	37	
	Ingesta	4		3	8	300	28	9
		4		3	8	600	52	
		4	12	3	8	600	52	9
	Ingesta	16		3	8	300	47	16
		16		3	8	600	75	
5	Feed				8	300	14	4–5
					8	600	27	
	Ingesta[3]	4		3	8	300	21	7–9
		4		3	8	600	54	
		16		3	8	300	31	10–13
		16		3	8	600	71	
	Ingesta[3]	4		3	8	300	30	8–9
		4		3	8	600	50	
		16		3	8	300	33	10–12
		16		3	8	600	61	
6[4]	Feed				8	300	31	10–12
					8	600	65	
	Ingesta	4		3	8	300	17	5–6
		16		3	8	300	8	4–6
		16		3	8	600	37	
7[4]	Feed				8	500	38	7–8
	Ingesta	4		1	8	500	35	7–8
		4	48	1	8	500	32	6–7

[1] Interval after last feeding when sample was withdrawn from rumen.
[2] Ground corn.
[3] Whole corn.
[4] Alfalfa hay ration.

tions are in accord with the findings of McElroy and Goss ('39) and offer a possible explanation as to why Johnson, Loosli and Maynard ('40) did not obtain improved growth in calves fed yeast or liver as supplements to a purified ration devoid of riboflavin.

The results of the thiamin studies presented in table 3 show that the dried ingesta of the rumen taken 12 or more hours after feeding contain less thiamin than the feed given the

TABLE 3

The comparative thiamin content of the feed and the dried ingesta of the rumen

TRIAL	MATERIAL	TIME[1] IN RUMEN	TIME INGESTA INCUBATED AT 37°C.	DAYS INGESTA COLLECTED	RATS	AMOUNT FED DAILY	AVERAGE GAIN OR LOSS IN WEIGHT	RATS WITH POLYNEURITIS
		hours	*hours*	*no.*	*no.*	*mg.*	*gm.*	*%*
1	Feed				6	400	20	
	Ingesta	12		1	6	400	−2	66
2	Feed				8	400	19	
	Ingesta	4		1	8	400	28	
		4	48	1	8	400	15	
3	Feed				8	400	21	
	Ingesta	16		3	8	400	10	30
		16	48	3	8	400	1	50
		16		9	8	400	2	
4	Feed				8	400	19	
					8	800	41	
	Ingesta	4		3	8	400	12	37
		4		3	8	800	50	
		4	12	3	8	800	34	
	Ingesta	16		3	8	400	9	25
		16		3	8	800	38	
5	Feed				8	400	21	
					8	800	72	
	Ingesta[2]	16		3	8	400	−10	37
		16		3	8	800	5	50
	Ingesta[3]	16		3	8	400		100
		16		3	8	800		100
7[4]	Feed				6	500	16	
	Ingesta	4		3	6	500	27	
		4	48	3	6	500	7	

[1] Interval after last feeding when sample was withdrawn from rumen.
[2] Whole corn. [3] Ground corn. [4] Alfalfa hay ration.

steer. The 4-hour ingesta samples, on the contrary, showed an increased thiamin content in two of the three trials. This suggests either that the thiamin was rapidly absorbed by the animal or that it was in part destroyed, since the incubation of the fresh ingesta in four trials for 12 or 48 hours resulted in a decreased thiamin content. Further studies are required to determine which of these possibilities is correct.

CONCLUSIONS

The results of these studies show that riboflavin is synthesized in the rumen of the steer when the animal is fed a ration of yellow corn, alfalfa hay, and a protein supplement.

Evidence was also obtained that the dried ingesta of the rumen of the steer fed exclusively on alfalfa hay contained less riboflavin than did the hay.

The data also indicate a greater thiamin content of the dried ingesta of the rumen removed 4 hours after feeding than in the feed, and a decreased content 12 or 16 hours after feeding.

LITERATURE CITED

BECHDEL, S. I., HANNAH E. HONEYWELL, E. ADAMS DUTCHER AND M. H. KNUTSEN 1928 Synthesis of vitamin B in the rumen of the cow. J. Biol. Chem., vol. 80, p. 231.

BECHDEL, S. I., C. H. ECKLES AND L. S. PALMER 1926 The vitamin B requirements of the calf. J. Dairy Sci., vol. 9, p. 409.

KICK, C. H., PAUL GERLAUGH, A. F. SCHALK AND E. A. SILVER 1938 Digestion in cattle—pH of the ingesta. Ohio Agricultural Experiment Station Bull. 592 (Annual Report), p. 105.

JOHNSON, P. E., J. K. LOOSLI AND L. A. MAYNARD 1940 Purified diet studies with calves. J. Dairy Sci., vol. 23, p. 553.

MCELROY, L. W., AND HAROLD GOSS 1939 Report on four members of the vitamin B complex synthesized in the rumen of the sheep. J. Biol. Chem., vol. 130, p. 437.

A STUDY OF THE NEED FOR COBALT IN DOGS ON MILK DIETS [1]

DOUGLAS V. FROST, C. A. ELVEHJEM AND E. B. HART
Department of Biochemistry, College of Agriculture, University of Wisconsin Madison

THREE FIGURES

(Received for publication September 3, 1940)

Cobalt was found to be essential in the nutrition of sheep by Underwood and Filmer ('35), Lines ('35) and of cattle by Neal and Ahmann ('37) and Filmer and Underwood ('37). In all of the above cases anemia was found to occur in the animals on natural rations grown in areas known to have a very low content of cobalt in the soil. Underwood and Elvehjem ('38) attempted to demonstrate a cobalt deficiency effect in rats on milk diets and concluded that, if a cobalt requirement exists at all, it must be less than 6 μg. per kilogram of body weight per day.

Since some of our growing dogs failed to attain normal blood values over several months' time with iron and copper therapy, we became interested in the possibility that cobalt might function at that point to stimulate hematopoiesis.

EXPERIMENTAL

Attempts to produce cobalt deficiency. Nutritional anemia was produced in a litter of six Beagle puppies by the method of Potter et al. ('38) and Frost and associates ('40). In this case the pups were obtained at 2 weeks of age and were placed with the mother on raised screens. Only cows' milk was fed

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Works Progress Administration.

to the mother for the next 2 weeks, at the end of which time the pups were weaned to milk. The development of anemia in these dogs was very rapid. The type of therapy varied with the different dogs, but two typical results are shown in figure 1. One of the dogs was given 20 mg. of iron and 2 mg. copper daily when the hemoglobin level reached 6 gm. per 100 cc. of blood. An immediate response occurred, but when the hemoglobin reached about 11 gm., there was little if any additional improvement. The level was maintained for about 10 weeks. When 4 mg. of cobalt per day were added in addition to the

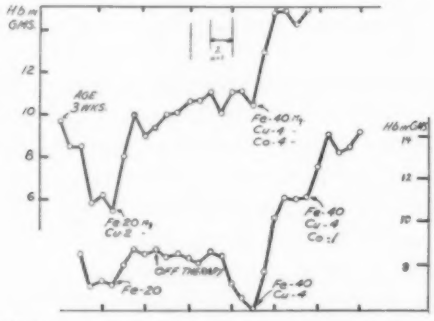

Fig. 1 The additive effect of iron, copper and cobalt in blood building in young dogs.

iron and copper, there was an immediate increase in hemoglobin and normal levels were reached. The other dog was given a preliminary treatment with iron alone to deplete the copper reserve. When iron and copper were given at levels of 40 mg. and 4 mg. respectively, the typical response was obtained but a plateau was again observed at 11 to 12 gm. hemoglobin. The dog showed a rapid increase in hemoglobin when 0.1 mg. of cobalt was given per day. The calculated hemoglobin production during the first 2 weeks of cobalt supplementation was many times that produced during any 2 weeks immediately preceding the cobalt feeding.

In order to check the apparent stimulating effect of cobalt for young dogs we repeated the experiment with another litter

of eight mongrel Spaniel pups. This litter was also obtained at 2 weeks of age with the mother and was weaned to cows' milk at 4 weeks of age. When the pups were 7 weeks old, they were quite uniformly anemic. The hemoglobin level in each case was between 4 and 5 gm. per 100 cc. of blood. Four pups were then selected for a comparison of the rate of hemoglobin building with and without cobalt. Each of one pair of dogs was fed 10 mg. of iron plus 2 mg. of copper daily and each of another pair was fed the same combination plus 0.5 mg. of cobalt daily. During therapy 6 cc. samples of blood were taken from the jugular vein every fourteenth day.

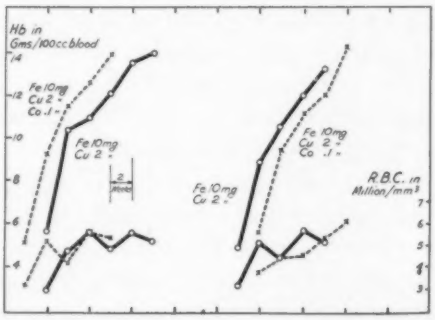

Fig. 2 Comparison of rate of blood building in young dogs fed iron and copper and iron, copper and cobalt from the beginning of therapy.

Figure 2 shows the uniformity of the blood picture as regards hemoglobin and red blood cell count in all cases. Hematocrit readings ranged from 16 to 19% at the start of therapy and 38 to 41% at the end of the 16 weeks period of therapy. As an added precaution against cobalt contamination in this experiment the ferric chloride was extracted with ether as follows: Analytical reagent iron wire (Mallinckrodt) was dissolved in C.P. concentrated HCl and extracted continuously in a Kutscher-Steudel apparatus with ether. Superoxol was used to oxidize the iron completely, but a small amount of ferrous chloride was added back to prevent the formation

of explosive ether peroxides during the extraction. The ether extract containing cobalt-free ferric chloride was freed from ether and made to the proper dilution by analysis.

Effect of hemorrhage. In order to determine whether or not a cobalt deficiency could be produced by repeated production of anemia through hemorrhage, or by long-continued nutritional anemia, the following experiments were performed. Two litter mate pups were made anemic by the usual procedure. One of them was given 10 mg. of iron, 2 mg. of copper, and 1 mg. of manganese daily for 13 weeks. At the end of this time the hemoglobin of the blood had reached a normal level. Therapy was discontinued and the dog was subjected to severe bleeding by phlebotomy. When anemia had again been produced, iron, copper and manganese were once more introduced. Again the anemia was rapidly and completely cured. A third anemia was produced by bleeding and was once more completely cured by iron, copper and manganese therapy.

The litter mate to the above dog was fed iron alone at weaning but was restricted to a total intake of 420 mg. of iron. The dog was then fed milk alone for several months and finally bled to produce a severe copper deficiency. At this point no response was shown to iron feeding. After 3 weeks copper was added to the iron therapy. The response to copper was not complete and the hemoglobin level remained at 11 gm. per 100 cc. of blood. The interesting observation was made at this time that the red blood cell count fell from 8 million per cubic millimeter of blood to 5.4 million during the period of copper therapy. When cobalt was added to the iron, copper and manganese at a level of 0.5 mg. per day, a sharp rise in hemoglobin and red blood cell count ensued. The hemoglobin level rose from 11 to 15.4 gm./100 cc. and the cell count from 5.4 to 9.4 M/c.mm. in a period of 3 weeks (fig. 3). Another dog made severely anemic by phlebotomy and depleted of copper showed an actual decrease in all blood values during iron and copper therapy, but exhibited a rapid increase when cobalt was added at a level of 0.1 mg. per day.

Addition of cobalt to dogs severely deficient in copper was without benefit even when adequate iron was given. In general the effect of cobalt seemed to be supplementary to that of iron and copper in those instances where copper and iron alone did not suffice to stimulate normal blood building.

Determinations of reticulocyte count, hematocrit per cent and hemoglobin were made at regular intervals throughout these studies to determine whether dogs on mineralized milk diets show normal blood cytology. The values obtained compare so closely with those described by Scarborough ('30), Mayerson ('30), and Morris et al. ('40) for large numbers of

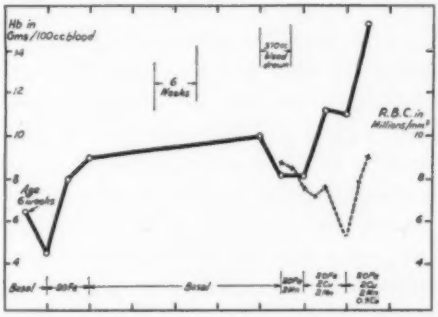

Fig. 3 Effect of cobalt addition to iron and copper therapy after long-continued nutritional anemia and hemorrhage.

dogs on regular kennel diets that it is not necessary to report them here. Let it suffice to say that the maintenance of hemoglobin at subnormal level in certain of our younger dogs is not to be confused with the normal physiological lag in arriving at adult hemoglobin levels shown by most species of animals in the rapid growing stage, and described for dogs by Morris et al. ('40). Comparison of our figures with those of Morris indicated that many of our dogs attained normal adult blood levels at a comparatively early age. Responses to cobalt appeared to be independent, so far as we could tell, of the age of the dogs.

DISCUSSION

The results of the above experiments indicate that a cobalt deficiency anemia is extremely difficult to produce uniformly in dogs on milk diets. The stimulating effect of cobalt which we have observed in certain dogs appeared quite real and unequivocal. Possibly this effect is non-specific and would be produced by other elements as well; however, the well-known capacity of cobalt to stimulate a polycythemic response in many species suggests that here we are dealing with a specific function of cobalt.

The cobalt content of milk is very low. Wright and Papish ('29) and Blumberg and Rask ('33) claim that milk is "spectrographically free" of cobalt. Underwood and Elvehjem ('38) used the method of Kidson, Askew and Dixon ('36) and obtained a mean of 11 μg. of cobalt per liter of milk in five analyses controlled by blanks and recovery tests. Results of several analyses which we made on 500 cc. samples of milk checked the results of Underwood and Elvehjem fairly well when the color comparisons were made visually. When the photometric estimation method of MacPherson and Stewart ('38) was used with the Evelyn photoelectric colorimeter, satisfactory recoveries of cobalt were not obtained on milk. Calcium is known to interfere with the accurate colorimetric determination of cobalt and this may explain the paucity of reports in the literature regarding the cobalt content of milk. For purposes of interpretation of our experiments we can safely say that the cobalt content of milk is low. Nothing is known of the possible variation in cobalt content of milks from different sources.

If we consider the cobalt content of milk to be from 10 to 20 μg. per liter, the cobalt intake of dogs on milk diet would be about 1.5 to 3 μg. per kilogram of body weight per day. Thus a 25 kg. dog might ingest as much as 0.075 mg. of cobalt daily from the milk alone. Since the daily requirement of a 25 kg. sheep has been placed at only 0.1 mg. per day, it appears that we may be operating at a level close to the minimal requirement. Only the use of purified diets or of foods from

cobalt deficient areas will permit a decision as to whether cobalt is generally essential for normal function of hematopoietic organs.

Use of cobalt in treatment of human anemia in infants by Kato ('37), in children by Waltner ('30) and in adults by Baxter ('39) and Cronin ('39) appeared successful only in the hands of the first author. In most of these cases rather large amounts of cobalt were used and in some of them the size of the dose would border on the predicted polycythemia-producing level for humans. A level of 0.15 mg. of cobalt per day is about the detectable polycythemia-producing level in rats. A polycythemic response in dogs has been obtained with a dose of 2 mg. per kilogram of body weight per day. The general biologic property of cobalt to produce polycythemia in animals may be quite unrelated to the essential role it plays in the nutrition of certain species. In any case the part played by cobalt in hematopoiesis is unique and continued study of its effects should throw some light on the intricate mechanisms involved in blood formation.

SUMMARY

In many cases iron and copper suffice with milk for normal hemoglobin building in dogs. The addition of small amounts of cobalt to iron and copper therapy stimulated hematopoiesis in certain dogs in which the rate of blood formation appeared unusually slow. About one-half of the dogs studied showed a cobalt deficiency as evidenced by hematopoietic responses to cobalt at a minimum level of 0.1 mg. per day.

LITERATURE CITED

BAXTER, C. R. 1939 Copper and cobalt in anemia. Brit. Med. J., p. 534.

BLUMBERG, H., AND A. S. RASK 1933 The spectrographic analysis of milk ashes. J. Nutrition, vol. 6, p. 285.

CRONIN, E. 1939 Copper and cobalt in anemia. Brit. Med. J., p. 643.

FILMER, J. F., AND E. J. UNDERWOOD 1937 Enzoötic marasmus. Further data concerning the potency of cobalt as a curative and prophylactic agent. Aust. Vet. J., vol. 13, p. 57.

FROST, D. V., C. A. ELVEHJEM AND E. B. HART 1940 Iron utilization in dogs on milk diets. J. Nutrition, vol. 19, p. 311.

KATO, K. 1937 Iron-cobalt treatment of physiologic and nutritional anemia in infants. J. Pediat., vol. 11, p. 385.

KIDSON, E. B., H. O. ASKEW AND J. K. DIXON 1936 Colorimetric determination of cobalt in soils and animal organs. New Zealand J. Sci. and Tech., vol. 18, p. 601.

LINES, E. W. 1935 The effect of the ingestion of minute quantities of cobalt by sheep affected with "Coast Disease." J. Coun. Sci. and Ind. Res., vol. 8, p. 117.

MACPHERSON, H. T., AND J. STEWART 1938 The photometric estimation of cobalt. Biochem. J., vol. 32, p. 763.

MAYERSON, H. S. 1930 The blood cytology of dogs. Anat. Rec., vol. 47, p. 763.

MORRIS, M. L., N. J. STELTON, J. B. ALLISON AND D. F. GREEN 1940 Blood cytology of the normal dog. J. Lab. Clin. Med., vol. 25, p. 353.

NEAL, W. M., AND C. F. AHMANN 1937 The essentiality of cobalt in bovine nutrition. J. Dairy Sci., vol. 20, p. 741.

POTTER, V. R., C. A. ELVEHJEM AND E. B. HART 1938 Anemia studies in dogs. J. Biol. Chem., vol. 126, p. 155.

SCARBOROUGH, R. A. 1930 The blood picture of normal laboratory animals. Yale J. Biol. Med., vol. 3, p. 359.

UNDERWOOD, E. J., AND J. F. FILMER 1935 Enzootic marasmus. The determination of the biologically potent element (cobalt) in limonite. Aust. Vet. J., vol. 11, p. 84.

UNDERWOOD, E. J., AND C. A. ELVEHJEM 1938 Is cobalt of any significance in the treatment of milk anemia with iron and copper? J. Biol. Chem., vol. 124, p. 419.

WALTNER, K. 1930 Therapeutische Versuche mit Kobalt. Acta. paediat., vol. 11, p. 438.

WRIGHT, N. C., AND J. PAPISH 1929 The inorganic constituents of milk. Science, vol. 69, p. 78.